Sustainable Cultivation of Microalgae for Biofuels: Supplying Organic Carbon and Other Nutrients from Low-cost Biomass

By
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ABSTRACT

Algal biofuels may provide a viable alternative to fossil fuels; however, this technology must overcome a number of hurdles before it can compete in the fuel market and be broadly deployed. These challenges include strain identification and improvement, nutrient and resource allocation and use, and the production of co-products to improve the economics of the entire system. In this study, the low-priced duckweed and filamentous algae were investigated as potential source of sugars for algal cultivation. The results showed that a certain part of sugars could be released from biomass and fed to algae as a source of carbon. The combination of acid/alkaline and enzyme pretreatment can be used for biomass hydrolysis, but still needs be optimized, since highest efficiency of sugar hydrolysis obtained is up to 50%. Different strains of algae were also cultured on acetic acid, butyric acid, propionic acid as different carbon sources under heterotrophic and mixotrophic conditions. Results obtained showed that acetic acid outcompeted other organic acids applied in this experiment as potential feedstock for algae cultivation mixotrophically. Strains including UTEX CS-01, UTEX 1230, UTEX 2714 displayed better growth than any other strains investigated in the experiments, with highest cells density of up to 3×10^8 cells/mL. This study shows that low-cost biomass and acetic acid can be potentially used as organic carbon source.

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CHAPTER 1 INTRODUCTION

Energy is always what supports us to survive on this splendid planet. However, with the advent of more technological industry, the global climate change and environmental pollution have risen to important issues that require us to open a "sustainable door" for future survival.

The global economy requires fuels to function, from producing diverse materials to providing the energy needed for lighting, heating and transportation. With our increasing population and expanding economy, incremental fossil fuel usage is taking place every minute. Data suggest that fossil fuel use will increase heavily, and competition for these limited resources will increase because many countries desire to improve their gross domestic product per capita. The shortage of limited energy sources is not the only dilemma we are facing. The increasing of atmospheric CO_2 concentration, and the potential for significant greenhouse gas-mediated climate change requires more and more attention [1]. These factors are driving the development of renewable energy sources that could replace fossil fuels, and allow greater access to energy resources for all nations, while greatly reducing carbon emissions into the atmosphere. The challenge that remains is to develop renewable energy industries that could operate sustainably and can be cost competitive with existing energy options. However, renewable technologies to supplement or replace liquid fossil fuels are still in their early developing stages. The International Energy Agency expects that biofuels will contribute only 6% of total fuel use by 2030, but could expand significantly if undeveloped petroleum fields are not accessed or if substantial new fields are not identified (Figure 1) [2].

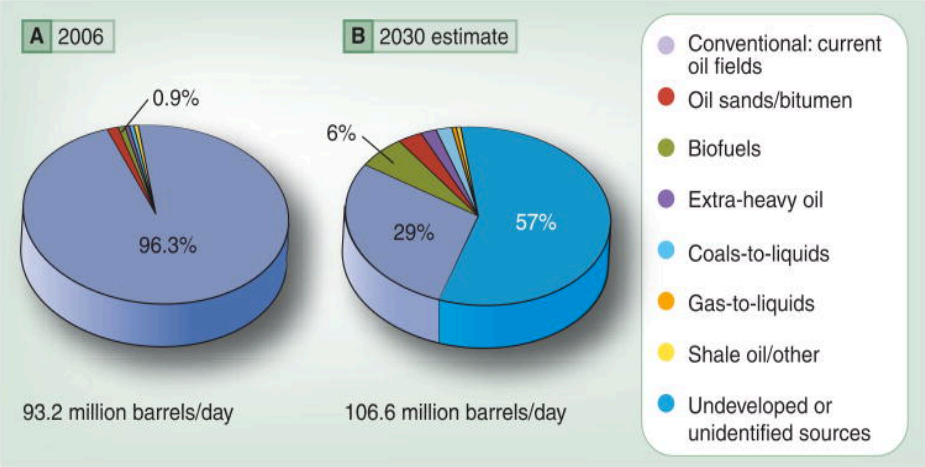


Fig. 1. Previous and predicted global petroleum sources

1.1 The emergence and definition of biofuels

The most promising sustainable alternatives are almost exclusively categorized under the moniker 'biofuels'. This term describes a diverse range of technologies that generate fuel with at least one component based on a biological system. Many countries and companies are investing heavily in biofuels for transport, motivated by concerns and opportunities related to global climate change, energy security, and rural development. Production targets and mandates for biofuels vary by countries, but many governments have adopted goals to substitute 10% or more of transportation demand for liquid fossil fuels with biofuels within 10 to 20 years.

In the US, the federal government passed the energy independence and security act (EISA) in 2007, which requires a gradual increase in the production of renewable fuels to reach 36 billion gallons per year by 2022. Furthermore, 28 states have passed their own mandatory renewable energy legislation. For example, Arizona and California will replace 15% and 20% of their electricity sales with renewable energy by 2020.

Biofuels can come from a wide variety of sources and can be roughly divided into four categories or "generations:"

- First generation biofuels are made from sugars, starches, oil, and animal fats that are converted into fuel using already-known processes or technologies. These fuels include biodiesel, bioalcohols, and biogasses, like methane captured from landfill decomposition.
- Second generation biofuels are made from non-food crops or agricultural waste, especially ligno-cellulosic biomass like switchgrass, willow, or wood chips.
- Third generation biofuels are made from algae or other quickly growing biomass sources.
- Fourth generation biofuels are made from specially engineered plants or biomass that may have higher energy yields or lower barriers to cellulosic breakdown or are able to be grown on non-agricultural land or bodies of water

The major technologies presently employed for biofuels begin with terrestrial plants and culminate with ethanol, whether this is corn starch-to-sugar-to-ethanol, or sugarcane sugar-to-ethanol. The regional success of some of these strategies is well noted; in particular, the sugarcane-to-ethanol production in Brazil [3]. However, the mass production of this first-generation liquid biofuels has resulted in a series of problems related to food prices, land usage, and carbon emissions. These strategies are functional at the small scale; however, as their use has increased, it is evident that they are not sustainable, owing to constraints on the productivity of biofuel crops such as water availability, the higher end of estimates for land-use. The challenge of meeting land needs for the expansion of biofuel production must be considered in the context of a

growing demand for food. Thus, the production of corn-based ethanol is supposed to be limited and the production of advanced biofuels should be increased. A number of hybrid strategies have been discussed or are currently being deployed. Examples of such strategies include conversion of cellulose to sugars for fermentation into fuel, and gasification of residual biomass into syngas that can be used to produce liquid fuels [4].

1.2 Microalgal-based bioenergy options

Algae are often defined as eukaryotic macroalgae and microalgae, but also prokaryotic photoautotrophic species such as cyanobacteria. Algal species grow in a wide range of aquatic environments, from freshwater through saturated saline. These groups contain species that efficiently make use of both CO_2 and organic carbon, e.g., glucose, as carbon sources, and also contribute to more than 40% of the global carbon fixation [5,6]. Algae can produce biomass very rapidly, with some species doubling in as few as 6 h, and many exhibiting two doublings per day [7,8]. Almost all algae have the capacity to produce energy-rich oils, and fortunately a number of microalgal species have been found to naturally accumulate high oil levels in total dry biomass [9]. While macroalgae are usually cultivated in their natural habitat, microalgae can be cultivated in dedicated cultivation systems, thus allowing us to put attention primarily on microalgal cultivation instead of macroalgal cultivation.

In recent years, biofuel production from microalgae is an appealing choice and becoming promising, because algae can grow nearly anywhere and algal biofuels can be blended with, or replace traditional fuels. As opposed to land-based biofuels produced from the land-based agricultural feedstock, cultivation of microalgae for biofuel does not

necessarily require agricultural land freshwater, and therefore competes less with food production than first generation biofuels.

1.2.1 Benefits of microalgal biofuels

Microalgae are a diverse group of single-celled organisms that have the potential to offer a variety of solutions for production of liquid transportation fuel. Combined with high productivity and potential wastewater treatment, biochemical content of microalgae and chemical conditions of their oil content can be influenced by manipulation of the growth conditions and subsequent quality control. In addition, microalgae have flexible metabolism that can be manipulated to increase biomass productivity and towards synthesis and accumulation of the high value products including pharmaceuticals, cosmetics. These advances can potentially reduce the biofuel processing cost.

Microalgae have less impact on our environment compared with other traditional sources of biomass used for biofuels [10]. They can be grown in open water (minimize land use) and are very efficient in removing nutrients from water (wastewater remediation). Depending on the species and cultivation conditions, algae can contain extremely high content of lipids or carbohydrates that are easily converted into a whole range of biofuels including biodiesel or bioethanol. Furthermore, the remaining biomass, composed mostly in protein and carbohydrate, may be processed into many other products such as: foods, chemicals, medicines, vaccines, minerals, animal feed, fertilizers, pigments skin creams. These beneficial characteristics make microalgae a platform with a high potential to produce cost-competitive biofuels.

1.2.2 Challenges for algal biofuel commercialization

The rapid growth rate (cell doubling time of 0.5-1 days), high lipid content (up to 50%-70% of the cell dry weight), smaller land usage (15–300 times more oil production than conventional crops on a per-area basis), and high carbon dioxide (CO_2) absorption and uptake rate have all been recognized as the reasons to invest significant capital to turn microalgae into biofuels. However, for microalgae to mature as an economically viable platform to offset petroleum and, consequently, mitigate CO_2 release, there are a number of hurdles to overcome ranging from how and where to grow and harvest these microalgae to improving oil extraction and fuel processing.

The algal biofuels production chain is outlined in Figure 2 [2] and shows that the major challenges include strain isolation, nutrient sourcing and utilization, production management, harvesting, co-product development, fuel extraction, refining and residual biomass utilization.

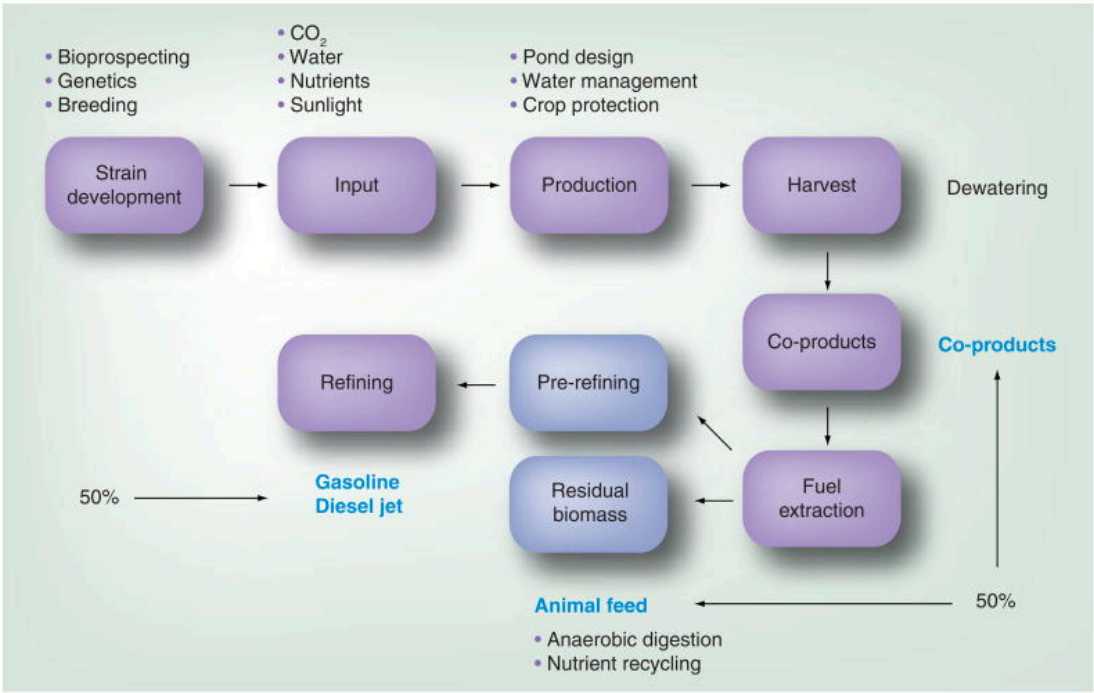


Fig. 2. Algal biofuels production chain

CHAPTER 2 LITERTURE REVIEW ON MICROALGAL CULTIVATION

2.1 Cultivation systems for microalgae

It is important to understand the basics of microalgae cultivation systems. A few microalgae have been harvested from the natural lakes because far more fuel is needed to collect and process wild algae than would be produced. Given that these practices are unlikely to sustain strong growth, only the cultivation of algae in man-made systems will be considered in this report. The main cultivation options described in detail in the main types are briefly presented below.

2.1.1 Open cultivation systems

The main large-scale algae cultivation system is the so-called raceway pond. These are simple closed-loop channels, which are usually 20-30 cm deep and made of concrete or compacted earth, often lined with water impermeable plastic film [11]. Water in there is motioned by a paddle wheel. Raceway ponds are designed for optimal light capture and low construction costs. However, it is difficult to control the process in such open systems: temperature is dependent on the weather and, given climatic conditions, large amounts of water cyclically evaporate or are added by rainfall. Furthermore, the open system paves a possible way for naturally occurring algae or algal predators to infiltrate the system and compete with the target algal species to be cultivated. Therefore a monoculture can only be maintained under extreme conditions, like high salinity (e.g. *Dunaliella*), high pH (e.g. *Spirulina*) or high nitrogen (e.g. *Chlorella*), thus generally limiting optimal growth and operate at a low algal concentration and making harvesting

more inaccessible. In conclusion, there is an important trade-off between a low price for the cultivation system and its production potential.

2.1.2 Closed cultivation systems

Many problems of open systems can be mitigated by establishing a closed system. Many configurations exist but all of them rely on the use of transparent plastic containers (usually tubes) through which the culture medium flows and in which the algae are exposed to light. Such a system is more expensive and therefore capital intensive if produced on a large scale, but allows a wider number of species to be cultivated, normally with a higher concentration and productivity. On the other hand, these systems suffer from high energy expenditures for mixing and cooling than open ponds and are also technically more difficult to build and maintain.

2.2 Phototrophic, heterotrophic and mixotrophic cultivation of microalgae

Today, the most common procedure for cultivation of microalgae is autotrophic growth. Because all microalgae are photosynthetic organisms, and many microalgae are especially efficient light convertors, microalgae are cultivated in naturally or artificially illuminated environments. Under autotrophic cultivation, the cells harvest light energy and use CO_2 as carbon source. Growing algae photosynthetically using only the energy of sunlight and assimilating CO_2 from polluted environments is highly appealing from a sustainability perspective; however, there are a number of inherent obstacles to this approach in terms of applying algae to produce high quality lipids useful as biofuels.

First of all, the cell densities obtained by our group and others for microalgae grown

phototrophically with sunlight alone are typically lower by as much as an order of magnitude when compared to mixotrophic (with organic carbon) or heterotrophic (exclusively organic carbon) cultivation methods. Phototrophic algal productivity is also severely limited by reduced light penetration at high biomass concentrations. A comparison of the growth dynamics for mixotrophic and heterotrophic growth versus phototrophic growth from our group is shown in Figures 3 and 4, respectively [12,13].

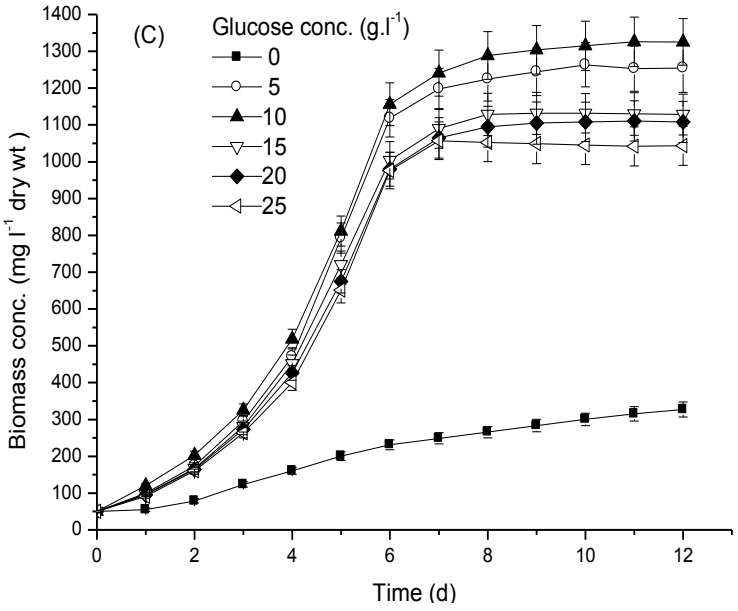


Fig. 3. Growth curves of *Chlorella sorokiniana* CCTCC M209220 under phototrophic and mixotrophic conditions

Secondly, the lipid content and therefore yields obtained by microalgae are often in the range of 15-25% of cell mass when induction occurs during photosynthetic operation. In contrast, yields of lipids that represent the precursors of biodiesel and jet fuel can rise to greater than 30% and approach 50% in our laboratories for microalgae that have been cultivated on organic carbon alone (heterotrophy) or together with CO_2 and sunlight (mixotrophically, see Figure 5). These processes can also yield high value products such

as TAG lipids (Figure 6). Thus, there is a significant incentive in terms of yields of liquid biofuels to grow microalgae in a mixotrophic or heterotrophic mode.

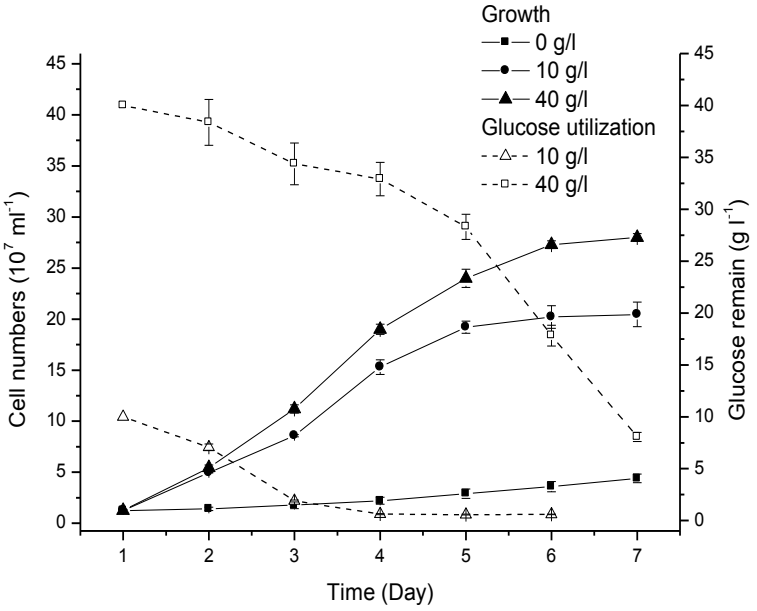


Fig. 4. Growth curves of *Chlorella sorokiniana* CCTCC M209220 under phototrophic and heterotrophic conditions

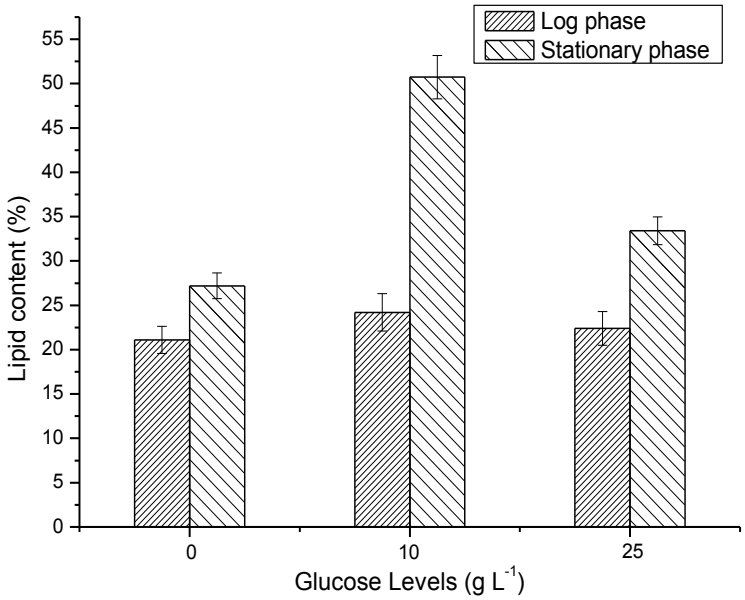


Fig. 5. Lipid content of *C. sorokiniana* CCTCC M209220 during different population growth periods under phototrophic and mixotrophic conditions

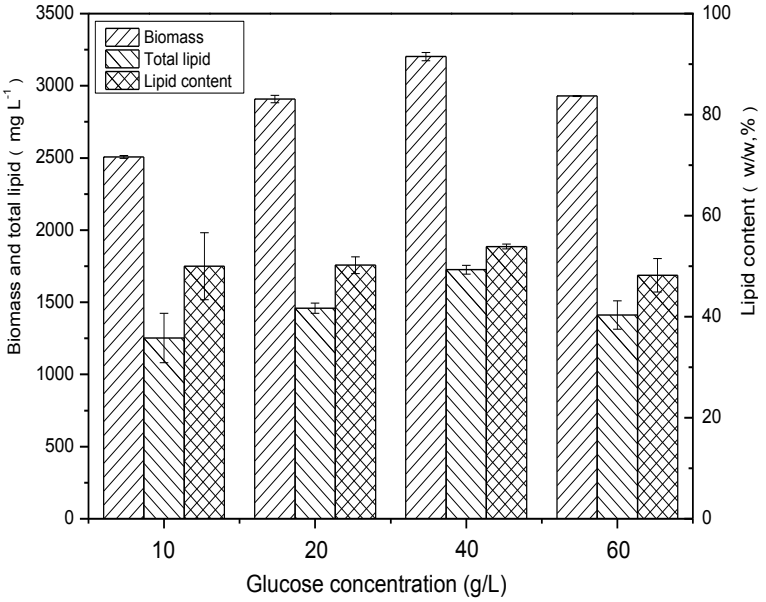


Fig. 6. Lipid content of *C. sorokiniana* CCTCC M209220 during different population growth periods under heterotrophic conditions

CHAPTER 3 MATERIALS AND METHODS

3.1 Experiment design

There are major economic disadvantages to growing microalgae on organic carbon. Indeed, feeding substrates such as glucose at levels required for high lipid yields will lead to a process that is commercially non-viable given the current cost of glucose at $0.3 per pound. Moreover, approximately 0.33 kg of nitrogen and 0.2 kg of phosphorus are required to generate 1 kg of algal biodiesel. In contrast, algae growing phototrophically are able to capture and sequester inexpensive and environmentally damaging CO_2, phosphate, and nitrate emitted from different industrial sources including polluted waters such as the Gulf of Mexico or the Chesapeake Bay. But limitations still exist in part due to our inability to optimize bioprocessing of algae from the laboratory to the commercial market place. In bioprocessing, our inability to understand microalgae at cellular levels limits our capacity to efficiently utilize CO_2, sunlight, and organic wastes for optimal cell growth and conversion to biofuel precursors. If microalgae are cultivated phototrophically at low densities, then the bioreactors will use large amounts of water, nutrient resources and incur high processing costs. If the algae are cultivated at too high a density, microalgal cells can be starved of sunlight, carbon, or other nutrients. In both cases, lipid yields are unreasonably low. Alternatively, if we could apply a source of organic carbon for algae at a lower price than commercial glucose and use nutrients from wastes, then microalgal biofuels processes could be made more economically viable. These microalgae may also be used to generate high yields of lipid products for biofuels.

3.1.1 Experiment I

- Apply thermochemical and enzymatic pretreatment to derive sugars and non-carbon

nutrients from low-cost biomass

- Test generated "growth medium" as substrate for microalgae at the mixotrophic and heterotrophic conditions

3.1.2 Experiment II

If the sugars generated from low-cost biomass could not be efficiently used as carbon source for microalgae cultivation to obtain high lipid content as potential biofuels, it's also possible to ferment the pretreated hydrolysate from biomass through anaerobic digestion (AD) process, thus converting those sugars into other organic carbon sources, like carboxylic acid. The experiment has two major tasks described as follows:

- Use biological fermentation to transform low-cost biomass into simple organic carbon compounds potentially utilizable by algae
- Screen and identify algal species able to utilize fermentation products under mixotrophic and heterotrophic conditions

3.2 Materials and methods for Experiment I

3.2.1 Biomass resources

Like plants, many algal species have rigid cellulose-based cell walls and accumulate starch as their main carbohydrate storage compounds as well as cell wall structure [14]. The majority of algal polysaccharides is potential biochemical feedstock and can be fermented to produce ethanol. Duckweed (Figure 7), often known as lemna minor, is the smallest and fast-growing flower plant on earth. As an aquatic plant, duckweed is commonly used to recover nutrients (e.g. Nitrogen and phosphorous) and toxic metals from agricultural and municipal wastewater. Growing high-starch duckweed for

conversion to feedstock for biofuel production is investigated in this report. To make comparison, filamentous algae (Figure 8) was also used as potential low-cost feedstock in the experiment. Both of the algae were purchased from Ven consulting, LLC in form of powder.

(a) (b)

Fig. 7. (a) (b) Duckweed (*Lemna minor*) grown using wastewater

(a) (b)

Fig. 8. (a) (b) Filamentous algal biomass generated in a Turf Scrubber treating wastewater

3.2.2 Thermochemical pretreatment

About 10.31 g of dry powder of duckweed and 9.14 g of dry powder of filamentous algae were separately dissolve in MQ water to prepare a 610 mL biomass solution in 1000 mL Erlenmeyer bottle with volatile solids around 10 g/L. The 610 mL biomass for each was divided into four parts to undergo different pretreatment: Thermo-Enzymatic pretreatment, Thermo-acid-enzymatic pretreatment, Thermo-alkaline-enzymatic pretreatment, Enzymatic pretreatment only.

(1) Thermo-acid pretreatment

Dilute acids break down the cellulose and hemicellulose polymers in cellulosic biomass to release individual sugars (Lee et al. 1999; Lenihan et al. 2010), which can be used to cultivate algae as carbon source. Acid pretreatment is used to degrade the biomass component into fermentable sugars by further acid hydrolysis or using enzymatic hydrolysis. The advantage of acid hydrolysis lies in that acids can penetrate lignin without any preliminary pretreated biomass, thus breaking down the cellulose and hemicellulose polymers to form individual sugar molecules [15]. Sulfuric and hydrochloric acid are most commonly used catalysts for hydrolysis of biomass.

In this experiment, the biomass was hydrolyzed at the level of 10g/L sulfuric acid (H_2SO_4) and 121°C for 30 minutes. The resulting solution was again divided into three parts. One was centrifuged at 42000 rpm for 10 minutes. Separate the solid part by centrifugation and filter the supernatant through 0.2μm filter with nylon membrane, purchased from Corning Incorporation, then saved in freezer for subsequent analysis. Another part was followed by enzymatic pretreatment and the rest was used as control during enzymatic incubation.

(2) Thermo-alkaline pretreatment

Alkaline pretreatment of lignocellulosic materials causes swelling, leading to decreased crystallinity, increased internal surface area, disruption of the lignin structure and separation of structural linkages between lignin and carbohydrates [16]. In our experiment, the prepared biomass was subjected to dilute alkaline hydrolysis containing 10 g/L Sodium Hydroxide (NaOH) at 121°C for 30 minutes. The resulting hydrolysate was also treated as described in acid pretreatment section,

3.2.3 Enzymatic pretreatment

The main obstacle of enzymatic pretreatment hydrolysis is that intercellular starch granules are bound within rigid cell walls (Libessart et al, 1995), thus a biomass pretreatment step is needed to break down the cell wall to release polysaccharides prior to enzymatic saccharification [17]. That's why we did thermo-chemical pretreatment first.

Two enzymes for enzymatic pretreatment, ACCELLERASE 1500 and ACCELLERASE XY were generously provided by the DuPont Company. ACCELLERASE 1500 enzyme complex contains a potent combination of enzymes which effectively modify and digest non-starch carbohydrates, the structural material of lignocellulosic biomass. ACCELLERASE 1500 is capable of efficiently and synergistically hydrolyzing lignocellulosic biomass into fermentable monosaccharides. ACCELLERASE XY hemicellulose enzyme complex is designed as an accessory product to supplement whole cellulases with xylanase activity and work synergistically to enhance various polysaccharide conversions for the lignocellulosic biomass processing industry. The enzymatic activity of ACCELLERASE XY is 20,000-30,000 ABXU/g, which is expressed in Acid Birchwood Xylanase Units, and the ACCELLERASE1500 is

expressed in carboxymethycellulose (CMC U) activity units of 2200-2800 CMC U/g and 450-775 pNPG U /g referring to Beta-Glucosidase activity.

The pH of the biomass after themo-chemical pretreatment was adjusted to 5.6 by using pH meter, followed by the addition of diluted ACCELLERASE1500 and diluted ACCELLERASE XY. The hydrolysis reactions were performed in capped (foam stopper) flasks and allowed to proceed for up to 4 hours at 50°C with continuous agitation on a Thermoshaker at 200 rpm. Incubations were terminated by cooling down after which the samples were centrifuged at 4200 rpm for 10 minutes. The supernatant were separated from the biomass pellets and frozen prior to analysis.

3.2.4 Analytical procedures

(1) Biomass compositional analysis

To determine the amount of biomass prepared for pretreatment, a 4 mL biomass suspension sample of each type (duckweed and filamentous algae) was dried in a pre-weighted aluminum dish at 105 °C for 2 h, and then dried in 550°C muffle furnace for 1 h. The final mass was expressed as volatile solid (VS).

(2) Sugar analysis

Total sugar was measured before and after the pretreatment of biomass by Phenol-sulfuric acid assay. Samples were filtered through a 0.2μm filter with nylon membrane and diluted if needed before transferring to 5 mL test tubes. To all the tubes, add 500μL of 4% phenol followed by 2.5 mL 96% sulfuric acid. Stand 10 minutes at room temperature. Measure the A_{490} of the sugar standards and unknown solutions to be analyzed. To calculate the concentration of sugar present in the samples, make a graph plotting A_{490} versus sugar weight of the sugar calibration standards.

(3) Nutrient analysis

Nutrients such as total nitrogen (TN) and total phosphorus (TP) were analyzed through HACH kits using TNT 826 featuring total nitrogen concentration of 1-16 mg/L N and TNT 844 featuring total phosphorus concentration of 0.5 to 5.0 mg/L PO_4-P.

3.2.5 Cultivation of microalgae by using pretreated biomass as feedstock

All the pretreated samples used for cultivating microalgae were neutralized nearly to pH 7 before inoculation. *Chlorella sorokiniana* (CS-01) was selected as target microalgae to be cultured. All the inoculation started at cells density of 10^4 cells/mL and microalgae were cultivated under mixotrophic and heterotrophic conditions. The cultivation setup is shown in Figure 9.

(a) (b)

Fig. 9. (a) (b) Microalgae cultivation using pretreated biomass as feedstock

3.3 Materials and methods for Experiment II

3.3.1 Algal strains and growth medium

Algal cultures were received from the Culture Collection of Algae at the University of Texas at Austin (UTEX) and were maintained on Bold Basal Medium (BBM) plates. The components of BBM media were as follows (per liter): 176 g KH_2PO_4, 75 mg K_2HPO_4, 75 mg $MgSO_4 \cdot 7H_2O$, 50 mg Tetrasodium EDTA, 31 mg KOH, 25 mg $CaCl_2 \cdot 2H_2O$, 25 mg NaCl, 11.4 mg H_3BO_3, 10 mg $FeSO_4 \cdot 7H_2O$, 8.83 mg $ZnSO_4 \cdot 7H_2O$, 1.84 mg H_2SO_4, 1.57 mg $CuSO_4 \cdot 5H_2O$, 1.44 $MnSO_4 \cdot H_2O$, 0.71 mg MoO_3, 0.49 mg $Co(NO_3)_2 \cdot 6H_2O$.

Table 1. List of tested algal strains

#	Strain
1	*Chlorella sorokiniana* (UTEX 1230)
2	*Chlorella sorokiniana* (CCTCC M209220)
3	*Chlorella sorokiniana* (UTEX 246)*
4	*Chlorella sorokiniana* (UTEX 2805)
5	*Chlorella vulgaris* (UTEX 2714)
6	*Chlorella vulgaris* (UTEX 395)
7	*Chlorella vulgaris* (UTEX 26)
8	*Chlorella kessleri* (UTEX 262)
9	*Chlorella kessleri* (UTEX 263)
10	*Chlorella kessleri* (UTEX 2228)
11	*Chlorella kessleri* (UTEX 2229)
12	*Chlorella protothecoides* (UTEX 29)
13	*Chlorella fusca var. vacuolata* (UTEX 252)
14	*Chlorella saccharophila var. saccharophila* (UTEX 247)
15	*Chlorella saccharophila var. saccharophila* (UTEX 2469)
16	*Chlorella sp.* (UTEX 2068)
17	*Chlorella sp.* (UTEX 2168)
18	*Chlorella sp.* (UTEX 2248)
19	*Scenedesmus dimorphus* (UTEX 1237)
20	*Scenedesmus dimorphus* (UTEX 417)
21	*Scenedesmus acutus f.alternans* (UTEX B 72)
22	*Scenedesmus obliquus* (UTEX 393)

Two media were employed. The inorganic medium was BBM media as described previously. The organic medium was of the same composition as the inorganic medium plus acetic acid, butyric acid and propionic acid individually, since these organic acids

are common intermediate produced during anaerobic digestion process. Table 2 lists the growth media with different organic acid for microalgal cultivation under different conditions. The media were prepared with MQ Water.

Table 2. List of different growth media for microalgal cultivation

Growth Media	Initial Concentration of Acid, mM
BBM only	0
BBM+Acetic acid	17.5
BBM+Butyric acid	17.5
BBM+Proponic acid	17.5

Acetic acid was purchased from Fisher Science with density of 1.05 g/mL. Butyric acid was in lab grade with chemical formula of $CH_3(CH_2)_2COOH$ and density of 0.96 g/mL. Propionic acid was also purchased from Fisher Science with density of 0.99 g/mL.

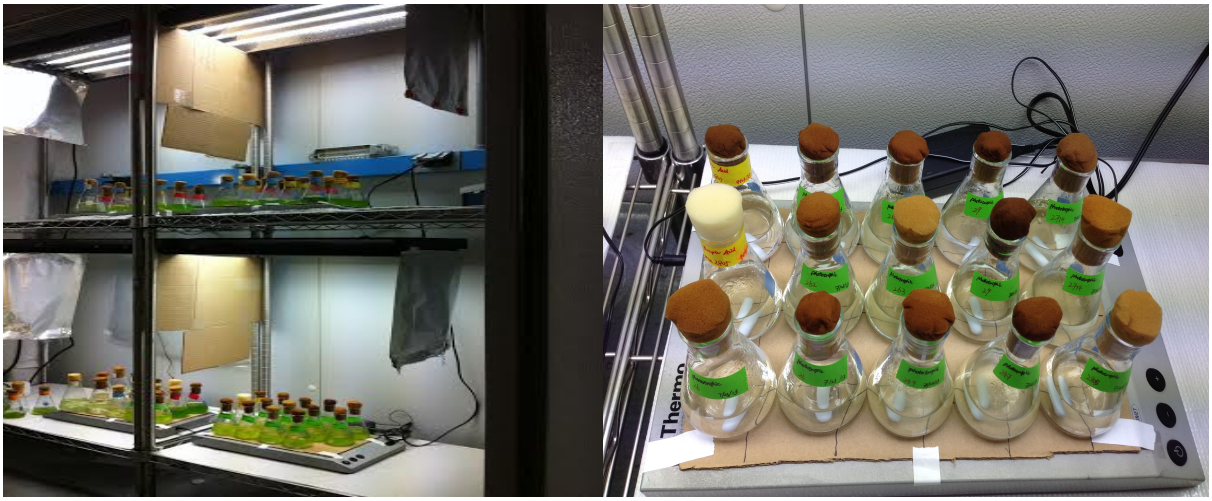
3.3.2 Cultivation experiments

Cultures were maintained on plates, and then scaled up in increasing volumes such that the cells were growing exponentially at each transfer. Before the start of the experiments, algal inoculum cultures were grown in a 125 mL flask containing 50 mL of autoclaved BBM medium using a fluorescent light source (8,000 Lum, 6,500 K, 14/10 hrs light/dark) and mixed by 20 mm magnetic stir bar at 350 rpm.

Before inoculation, growth media prepared were neutralized to pH 7.0 and contamination check was needed for algal inoculum. A few drops of inoculum were taken from the flasks onto the plates especially prepared for contamination check and spread evenly through autoclaved plastic beads. Plates were sealed with the Parafilm and placed in the AD Chamber without light penetrating through. After 24 h, plates were taken out and replaced in the Chamber where the algae were cultivated. Check the plates carefully

3-4 days after replacement. The plates with suspicious colonies were abandoned and those that looked uncontaminated were selected as algal strains to cultivate.

For phototrophic and mixotrophic cultivation, microalgae were cultivated in 150 mL bioreactor (Pyrex flasks) in constant temperature chamber under 22.5 °C. Magnetic stir bar was added into each bioreactor and the bioreactors were placed on magnetic stir plate to keep mixing at 350 rpm, as shown in Figure 10. Fluorescent grow light system is used as illumination facility, with the condition of 8,000 Lum, 6,500 K, 14 hours light and 10 hours dark. Heterotrophic cultivation of algae (Figure 11) were carried out in a covered incubation shaker at 23 °C with 200 rpm mixing speed.



(a) (b)

Fig. 10. (a) (b) Phototrophic and mixotrophic cultivation of microalgae

(a) (b)

Fig. 11. (a) (b) Heterotrophic cultivation setup for microalgae

Monoculture of each algal strain was added into two bioreactors in duplicate with same growth media and the algae solution started with cells density of 10^4 cells/mL. For each algal strain cultivated in the experiment, the growth media listed above were all applied individually as carbon source under different conditions.

3.3.3 Determination of Algal Growth

The cell density of algal solution was determined by using Viacount provided by GUAVA TECH, which is a cells counting machine with specialized operation software. The ViaCount assay uses a proprietary mixture of two DNA binding dyes to provide sensitive, accurate detection of viable, apoptotic, and dead cells. A membrane-permeant dye stains all nucleated cells, leaving cellular debris unstained so that you can focus on whole cells. A membrane-impermeant dye stains only damaged cells (i.e. those with breaches in the plasma membrane), thus indicating apoptotic and dying cells. About 0.5 mL samples of algal solution were collected into 1.5 mL centrifuge tubes everyday

during cultivation and went through Viacount to determine the cells density. The cultivation was stopped when algal growth reached static status.

3.3.4 TSS/VSS analysis

At the end of cultivation, the rest of the algal solution was collected and went through TSS/VSS measurement: Filter through Glass Fiber Filter, purchased from Millipore, with pore size of 1.0 μm. Dry the samples at 105 °C oven for 2 h, followed by another one hour in 550°C muffle furnace.

CHAPTER 4 RESULT AND DISCUSSION

4.1 Result and discussion for Experiment I

4.1.1 Sugar analysis

For filamentous algae as the source of organic carbon and nutrients for cultivation, the hydrolysis proceeded within 1 hour with 0.65 mL of diluted (1:2) ACCELLERASE 1500 and 0.65 mL of diluted (1:5) ACCELLERASE XY. About 1.1, 1.0, 0.5 mg/mL of sugars was released after Thermo+Enzyme, Thermo+Acid+Enzyme and Thermo+Alkaline+Enzyme pretreatment respectively. The enzymatic pretreatment alone was found to be insufficient for hydrolysis of the untreated filamentous algal biomass (Figure 12). For Alkaline treated biomass, the enzymatic hydrolysis reached a maximum at about 1.5 h; however, for Thermo and Acid treated, the hydrolysis and release of sugars occurred over four hours of the experiment. Residence time seemed to maintain a positive effect over saccharification process (Figure 12). The Thermal pretreatment combined with enzymatic hydrolysis gave the best yield of sugars compared with other pretreatments.

The highest yield of released sugars from duckweed for each pretreatment was achieved almost at the same time (with 1.2 h) by Thermo+Enzyme pretreatment (0.9 mg/mL), Thermo+Acid+Enzyme pretreatment (1.7 mg/mL), Thermo+Alkaline+Enzyme pretreatment (1.0 mg/mL). The blank incubation (without acid or alkaline pretreatment) released negligible amount of sugars (Figure 13). The results demonstrate that in enzyme, the bulk of duckweed sugars can be hydrolyzed and released over a certain period, from which, Thermo+Acid+Enzymatic pretreatment was most effective in sugar release.

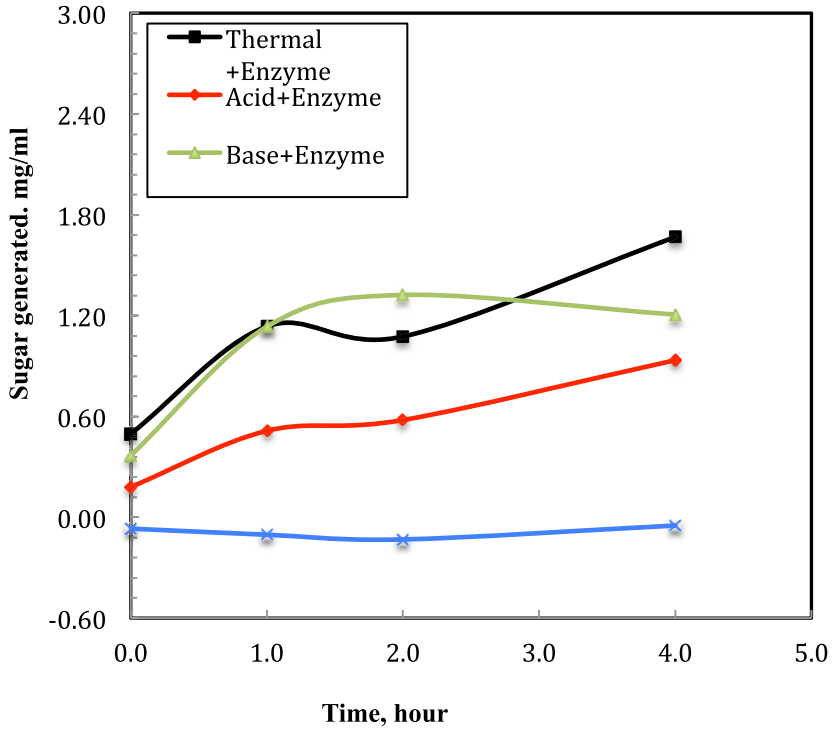


Fig. 12. Sugar concentration of pretreated biomass for filamentous algae during incubation

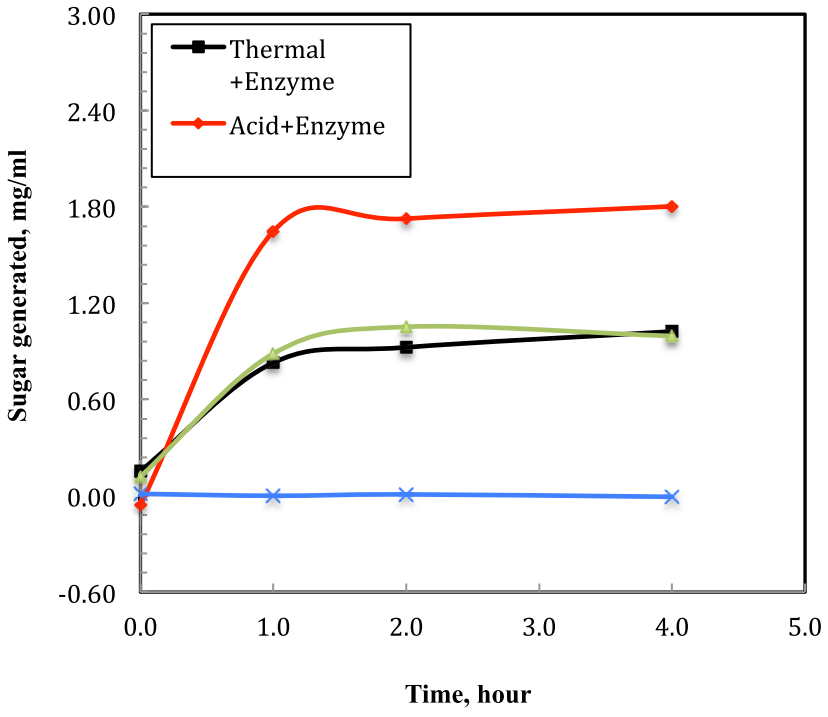


Fig. 13. Sugar concentration of pretreated biomass for duckweed during incubation

The final result showed that acid or alkaline pretreated biomass have improved enzymatic digestibility compared to untreated samples for both filamentous algae and duckweed. The combination of dilute acid or alkaline treatment with enzymatic hydrolysis can provide more significant saccharification of biomass such as filamentous algae and duckweed. Pretreatment of feedstock, especially those containing cellulose and hemicellulose, and subsequently hydrolysis of polysaccharide into monosaccharide are key steps in the production of biofuels.

However, the levels of biomass enzymatic digestibility were insufficient. The efficiency of sugar hydrolysis was in the range of 15-24% for filamentous algae and 25-53% for duckweed (Figure 14,15). The acidic or alkaline pretreatment of biomass caused increasing release of sugars during the following enzymatic hydrolysis, but the fraction of released sugar was still quite low.

Several pretreatment parameters may impact on the biomass hydrolysis efficiency including pH, temperature, concentration and types of enzyme. The influence and importance of these parameters need to be investigated in order to optimize the yield of sugars.

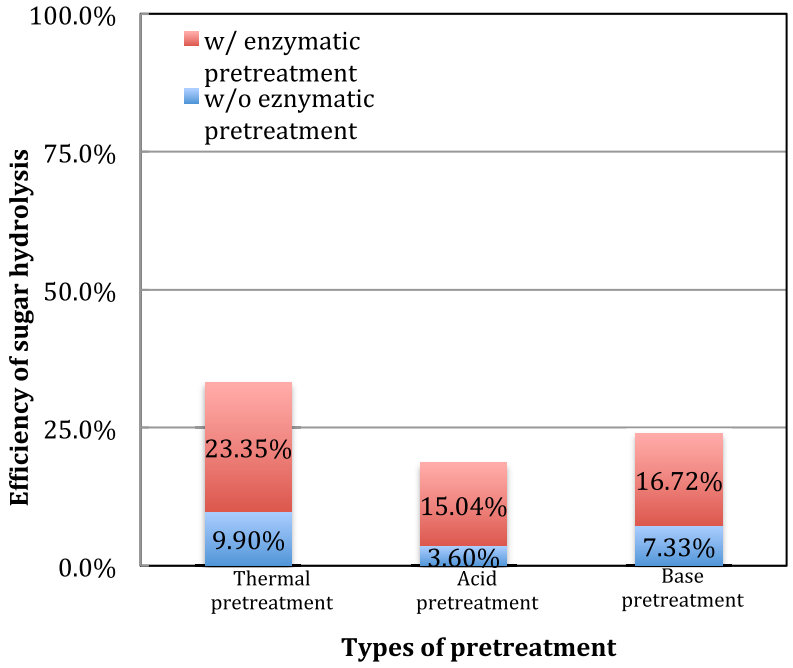


Fig. 14. Efficiency of sugar hydrolysis of pretreated biomass for filamentous algae during incubation

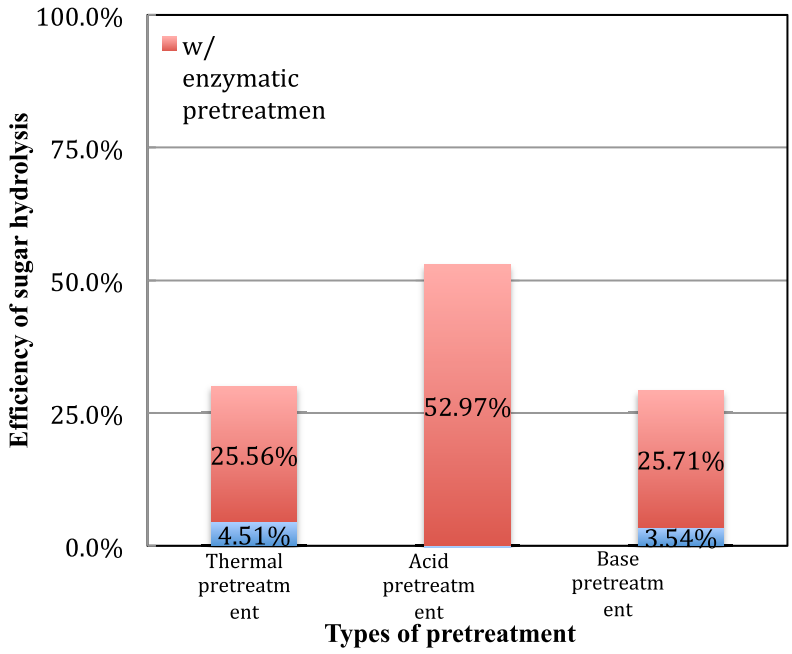


Fig. 15. Efficiency of sugar hydrolysis of pretreated biomass for duckweed during incubation

4.1.2 Nutrient analysis

Many nutrients are required for the prolific algal growth but Nitrogen and Phosphorous are especially important. Therefore, the total Nitrogen (TN) and Total Phosphorous (TP) in the hydrolysate were analyzed after enzymatic pretreatment to validate if they meet the nutrient need for algal cultivation. The results are shown in Table 3 in comparison with the nutrient composition in modified BBM growth media.

Table 3 Total nitrogen and total phosphorus concentration in pretreated biomass

Biomass	Standard mBBM	Pretreatment for filamentous Algae			Pretreatment for duckweed		
	with 5g/L glucose	Thermal	Acid	Base	Thermal	Acid	Base
Total nitrogen g/L	0.0564	0.152	0.202	0.311	0.294	0.328	0.490
Ratio of C/N	88.6525	12.377	21.149	5.449	4.451	8.301	3.038
Total phosphorus g/L	0.0794	0.026	0.047	0.056	0.077	0.107	0.093
Ratio of C/P	62.9723	73.173	90.216	30.149	17.016	25.340	15.989

Chlorella can grow on various organic and inorganic nitrogen compounds. The main nitrogen sources for growth of *Chlorella* are ammonium and nitrate salts [18]. When ammonium and nitrate are supplied together, the microalgae preferentially assimilate ammonium–nitrogen first, which is incorporated into the organic compounds produced by the microalgae [18]. Enhanced cellular nitrogen uptake was notable when the medium was supplemented with a source of carbon. These results can be explained by the fact that microalgae need more nitrogen when assimilating carbon [19]. From the comparison of TN between mBBM and pretreated biomass, it is clear that nitrogen is sufficient for algal growth. TP seems sufficient for pretreated duckweed rather than

filamentous algae. Given *Chlorella* is more like N limited, no extra P was added into the pretreated filamentous algae.

C/N, C/P ratio is another important parameter to access algal cultivation. In our experiment, C/N and C/P ratios are lower in pretreated biomass than that of mBBM, indicating that nitrogen and phosphorous is not the rare limiting during cultivation.

4.1.3 Cultivation of *Chlorella sorokiniana* (CS-01) using biomass hydrolysate

Chlorella sorokiniana (CS-01) was cultured in this experiment. The present investigation revealed the extent to which CS-01 can grow mixotrophically and heterotrophically by utilizing sugars hydrolyzed from pretreated biomass. The growth curves for CS-01 cultivated in the media resulting from filamentous algae after enzymatic hydrolysis are shown in Figure 16 and 17. The growth curves for CS-01 have the similar progression: 4 days of logarithmic phase, followed by stationary phase. The highest cells density (3.00×10^8 /mL) of CS-01 was achieved in the growth media derived by Thermo+Enzymatic pretreatment under mixotrophic cultivation. Thermo+Alkaline+Enzymatic pretreated biomass also provided a good carbon source for CS-01 cultivation. The cells density was comparative to that in cultures cultivated in Thermo+Enzymatic pretreated biomass.

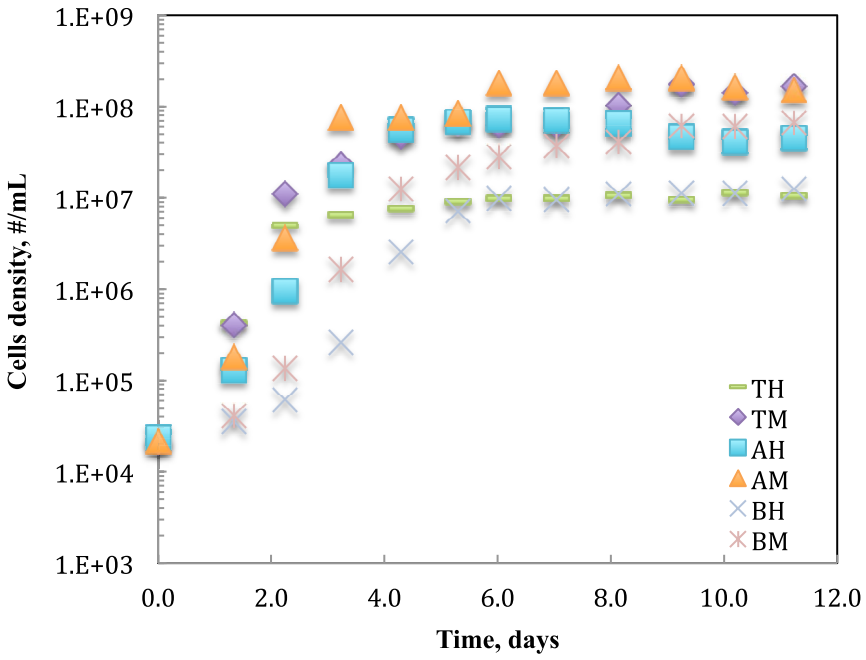


Fig. 16. Growth curves for *Chlorella Sorokiniana* using thermochemical pretreated filamentous biomass as feedstock

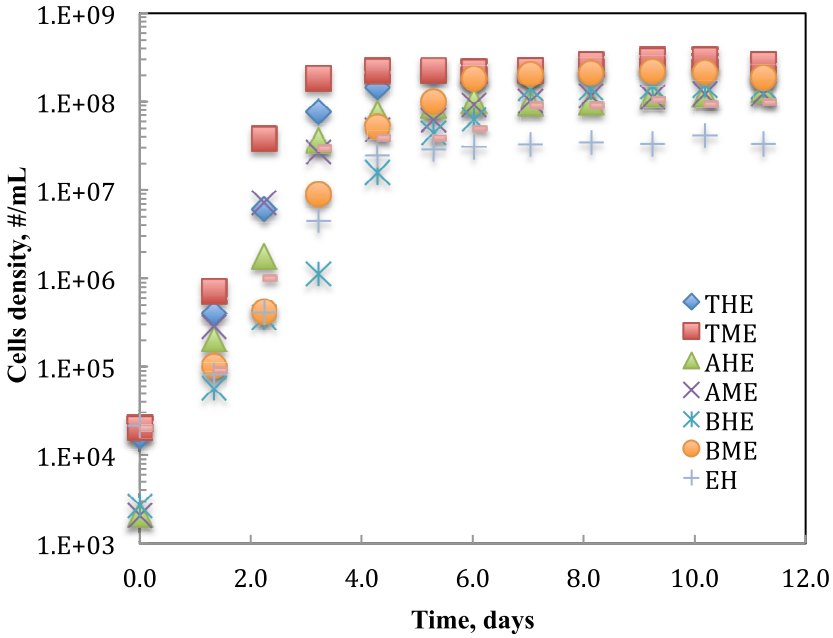


Fig. 17. Growth curves for *Chlorella Sorokiniana* using thermochemical and enzymatic pretreated filamentous biomass as feedstock

The growth curves for CS-01 cultivated in the pretreated duckweed, are shown in Figure 18 and 19. The highest cells density (2.80×10^{8} /mL) appeared after 4 days of cultivation in Thermo-Acid-Enzymatic pretreated duckweed. It is not surprising to see such a result which can be also estimated from previous result on sugar analysis.

However, microalgae stopped growing after 4 days almost in all types of growth media, which may indicate that one of the nutrients, become a rate limiting,. It could be nitrogen since the total nitrogen content in the growth media was related to the final density of the microalgae: higher N contents resulted in higher final cells density. It would be important to conduct additional experiment with increased concentration of the organic carbon or nitrogen to determine the rate-limiting nutrient.

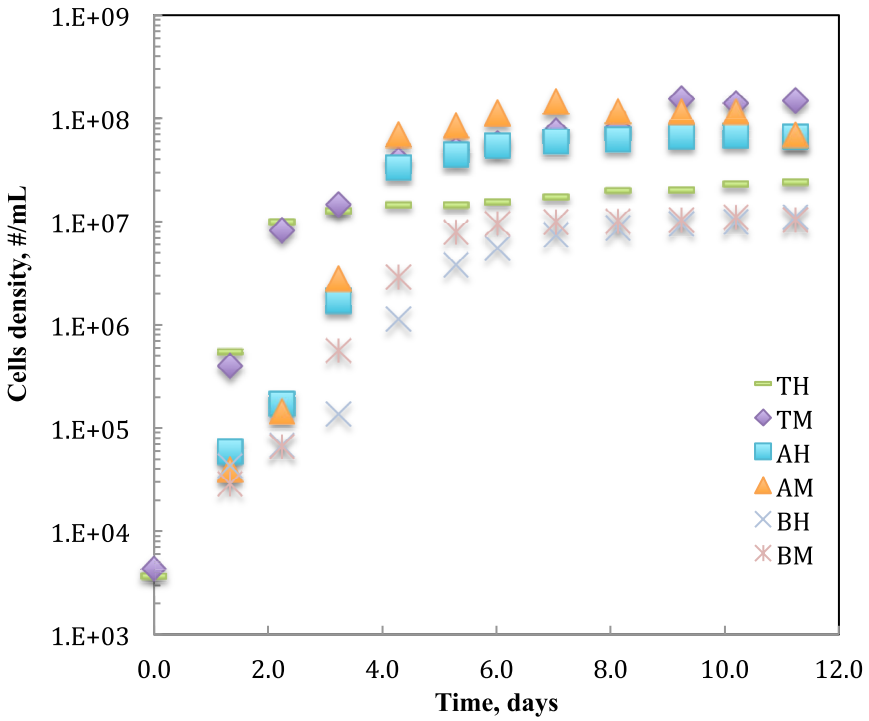


Fig. 18. Growth curves for *Chlorella Sorokiniana* using thermochemical pretreated duckweed biomass as feedstock

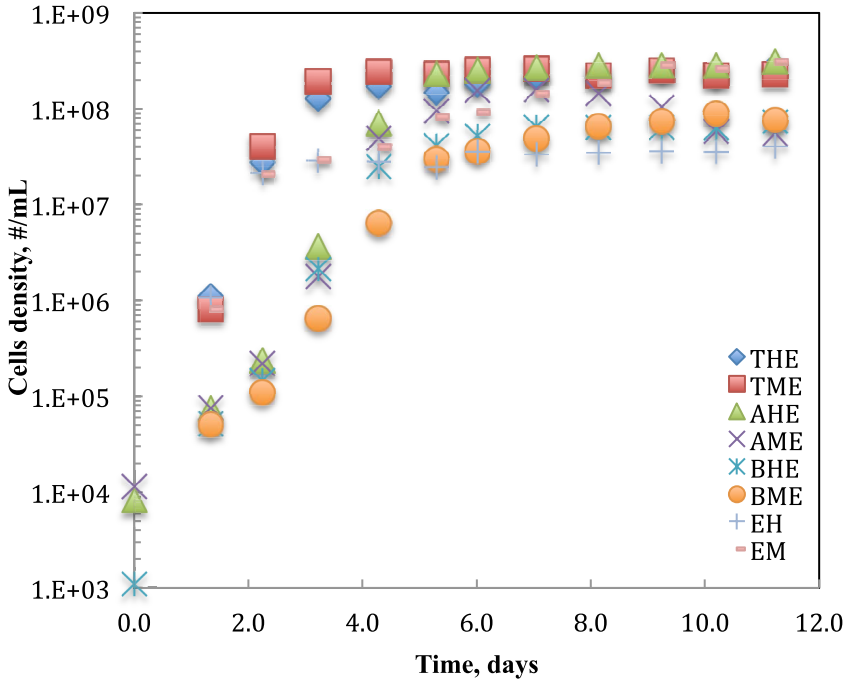


Fig. 19 Growth curves for *Chlorella Sorokiniana* using thermochemical and enzymatic pretreated duckweed biomass as feedstock

4.2 Result and discussion for Experiment II

4.2.1 Screen of algal strains in defined growth media

In this experiment, two batches of algal cultivation were conducted. The first batch experiment features a variety of algal strains including UTEX 417, UTEX 262, UTEX 263, UTEX29, UTEX 2714, UTEX 26, UTEX 247, UTEX 2805, UTEX B72, UTEX CS-01, UTEX 1230, UTEX 2248, UTEX 393, UTEX 1237, UTEX 246, UTEX 395. The growth curves are shown in Figure 20. The rest of the growth curves are presented in the Appendix. The second batch experiment repeated some of the algal strain grown in the first batch with addition of two new strains (UTEX 246, UTEX 2228) and added lactic

acid (with density of 1.209 g/mL) as another organic carbon source. The only difference in preparation for growth media lies in that pH was adjusted in each individual flask for algal cultivation in Batch I, while pH was neutralized to 7.0 in 4000 mL flasks before distribution into 150 mL flasks in Batch II.

The growth of *Chlorella* cultivated under phototrophic, heterotrophic and mixotrophic conditions on different media are shown in Figure 20. Horizontal axis presents the cultivation time in days, and vertical axis presents the average cells density of two duplicate bioreactors. The open diamonds illustrated the algal growth under phototrophic cultivation condition. All solid symbols indicate the mixotrophic condition and the other open symbols indicate the heterotrophic condition. Inverted triangles refer to growth curve obtained from acetic acid as carbon source, regular triangles for butyric acid and circles for propionic acid. For almost all the algal strains, acetic acid seemed to be the best organic carbon source to be assimilated by *Chlorella* under mixotrophic cultivation in spite of some exceptions such as UTEX 395, UTEX 417.

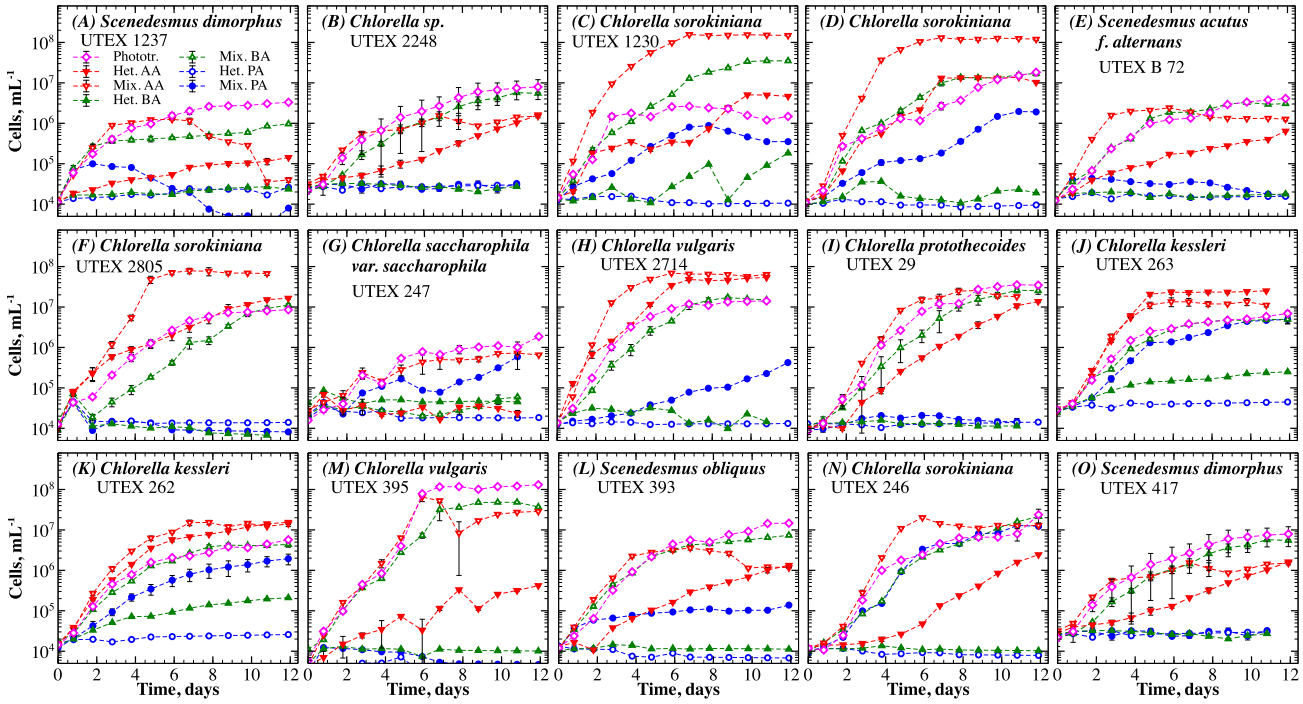


Fig. 20 Algal strains screening cultivation curves

As seen in Figure 4.9, the microalgal growth rate was highest on the BBM medium with acetic acid under mixtrophic conditions for microalgal strains including UTEX 1230, UTEX CS-01, UTEX B72, UTEX 2805, UTEX 262, UTEX 263, followed by phototrophic cultivation on BBM media only. The highest final cell density reached up to 3×10^8 /mL. A statistically significant difference in growth was observed between acetic acid and butyric acid or propionic acid as carbon source under heterotrophic cultivation, in terms of most of algal strains. Furthermore, nearly no growth was observed when algae were cultured in butyric acid and propionic acid heterotrophically. During cultivation of UTEX 29, longer log phase was observed in heterotrophic cultivation by using acetic acid as carbon source, probably due to longer adaptation time of the microalgae to the nutrients under heterotrophic condition as compared to phototrophic or mixotrophic

cultivation. Although some strains were able to grow in the presence of butyric acid and propionic acid at mixotrophic condition, the growth rate and final cells density were still lower or at most comparative to that of phototrophic cultivation.

The high cells densities of mixotrophic cultures demonstrate that the growth-stimulating effects of light and CO_2 utilization in mixotrophic cultures were as strong as the effects of acetic acid [21], which could be due to higher energy availability contributed by aerobic respiration coupled with catabolism of carbohydrates present in the medium along with photosynthesis. Similar results were reported that mixotrophic *Chlorella* produced higher biomass concentrations than those under heterotrophic and phototrophic conditions [20]. Algal trains like UTEX 2248, UTEX 395 and UTEX 417 grew to high cells density when cultivated phototrophically compared to other conditions, probably owing to that they cannot efficiently utilize light in the presence of carbon sources.

Some algal strains (UTEX CS-01, UTEX 1230, UTEX 2714) maintained green color while cultivated heterotrophically. This was distinctly different from some other microalgae at heterotrophic conditions, where the cultivation broth turned yellow probably due to the loss of chlorophyll in the absence of light.

4.2.2 TSS/VSS analysis

The final concentration of the volatile solids in the culture for different algal strains is shown in Table 4. If comparing the VSS results to cells density we acquired at the end of cultivation, we could see a respective correlation between VSS and cells density. For most of the algal strains, mixotrophic cultivation on acetic acid gave us highest VSS among others.

Table 4. (a) (b) Results from TSS/VSS analysis

(a)

Cultivation Condition	Volatile Solid in Biomass, mg/mL						
	UTEX 417	UTEX 262	UTEX 263	UTEX 29	UTEX 2714	UTEX 247	UTEX 2805
Phototrophic	0.3126	0.2788	0.2800	0.2591	0.3400	0.3036	0.2345
Heterotrophic on Acetic Acid	0.0964	0.2755	0.3100	0.2545	0.2518	/	0.2182
Mixotrophic on Acetic Acid	0.4680	0.8700	0.8517	0.5509	0.8457	0.3500	0.7350
Heterotrophic on Butyric Acid	/	/	/	/	/	/	/
Mixotrophic on Butyric Acid	0.3173	0.4837	0.4818	0.2891	0.5271	0.1791	0.2609
Heterotrophic on Propionic Acid	/	/	/	/	/	/	/
Mixotrophic on Propionic Acid	/	0.2709	0.4182	/	0.0964	0.2655	/

(b)

Cultivation Condition	Volatile Solid in Biomass, mg/mL							
	B 72	CS-01	UTEX 1230	UTEX 2248	UTEX 393	UTEX 1237	UTEX 246	UTEX 395
Phototrophic	0.3300	0.3500	0.3230	0.3433	0.3240	0.4240	0.3717	0.2480
Heterotrophic on Acetic Acid	0.1530	0.1980	0.2260	0.2390	0.0720	0.0460	0.1180	0.0520
Mixotrophic on Acetic Acid	0.5690	0.7483	0.8100	0.5186	0.4717	0.4980	0.4267	0.5800
Heterotrophic on Butyric Acid	/	/	/	/	/	/	/	/
Mixotrophic on Butyric Acid	0.3750	0.4150	0.4563	0.4120	0.3420	0.2510	0.3700	0.2713
Heterotrophic on Propionic Acid	/	/	/	/	/	/	/	
Mixotrophic on Propionic Acid	0.0380	0.1070	0.1000	0.1990	0.0360	/	0.2133	/

CHAPTER 5 CONCLUSION AND FUTURE PLAN

5.1 Conclusion

Our result obtained from the experiments and data analysis show that combined acid/alkaline and enzymatic pretreatment of duckweed and filamentous algae biomass is a more effective hydrolysis method than either acid/alkaline or enzymatic pretreatment alone. Also, the released sugar and other nutrients including nitrogen and phosphorus can be used as carbon sources for microalgal cultivation under both heterotrophic and mixotrophic conditions. The addition of the low-cost hydrolysate significantly enhanced the growth rate and final cell density of the several microalgae including CS-01, UTEX 1230 and UTEX 2714. However, the efficiency of biomass hydrolysis and the utilization rate of hydrolyzed sugars are not maximized. Additional experiments are necessary in order to optimize the hydrolysis process and improve microalgal cultivation processes.

In addition, most of the *Chlorella* strains could be cultivated mixotrophically and heterotrophically on acetic acid as organic carbon source. Strains including CS-01, UTEX 1230, UTEX 2714 gave highest cells density after 11 days cultivation, to which we should pay more attention. They are potential algal strains we might acquire higher lipid content from. In contrast, addition of propionic, lactic or butyric acids either didn't improve or even inhibited the algal growth rate at mixotrophic conditions.

5.2 Future plan

(1) Optimize the low-cost biomass pretreatment parameters including pH, temperature, acid/alkaline concentration and enzyme dose;

(2) Determine the sugar composition of biomass hydrolysate;

(3) Scale up microalgal cultivation using hydrolyzed low-cost biomass as source of organic carbon and other nutrients;

(4) Monitor consumption of organic carbon and nutrient (N, P) by microalgae during cultivation in order to optimize the medium C/N and C/P ratio;

(5) Determine the lipid content of harvested microalgal biomass;

(6) Examine acid incorporation and pathway utilization through metabolic flux analysis;

(7) Apply stoichiometric models of cellular metabolism to optimize the bioreactor performance for lipids production through mixotrophic/heterotrophic microalgae cultivation on acetic acid.

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APPENDIX : Growth curves of *Chlorella* strains cultivated on acetic acid, butyric acid, propionic acid and lactic acid under phototrophic, heterotrophic and mixotrophic condition

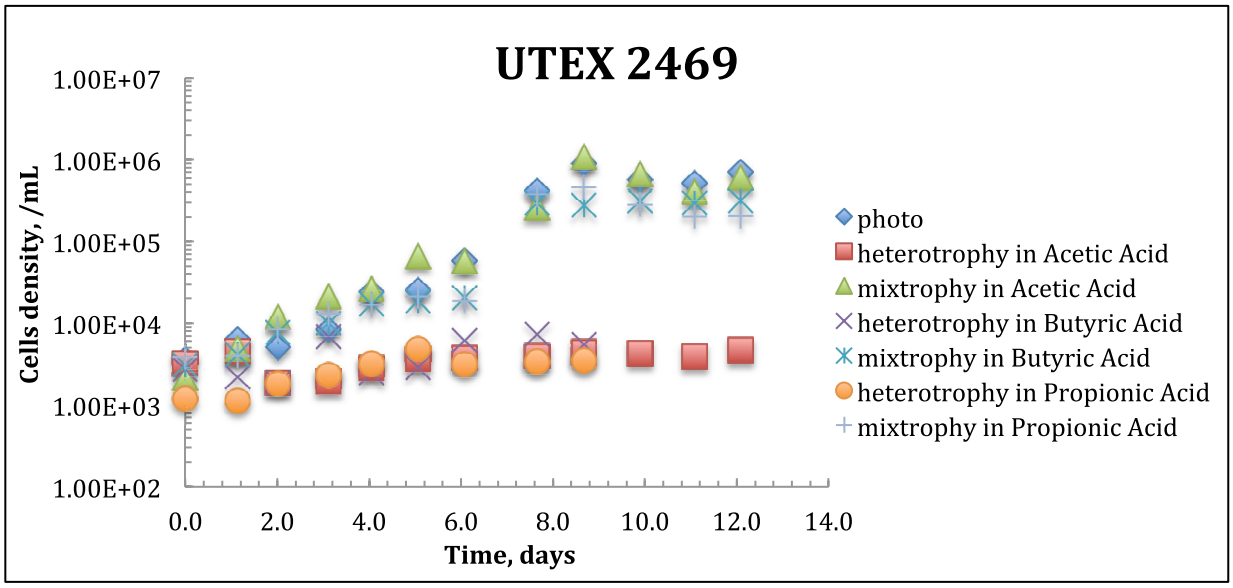

Figure 21. Growth curves for UTEX 2469

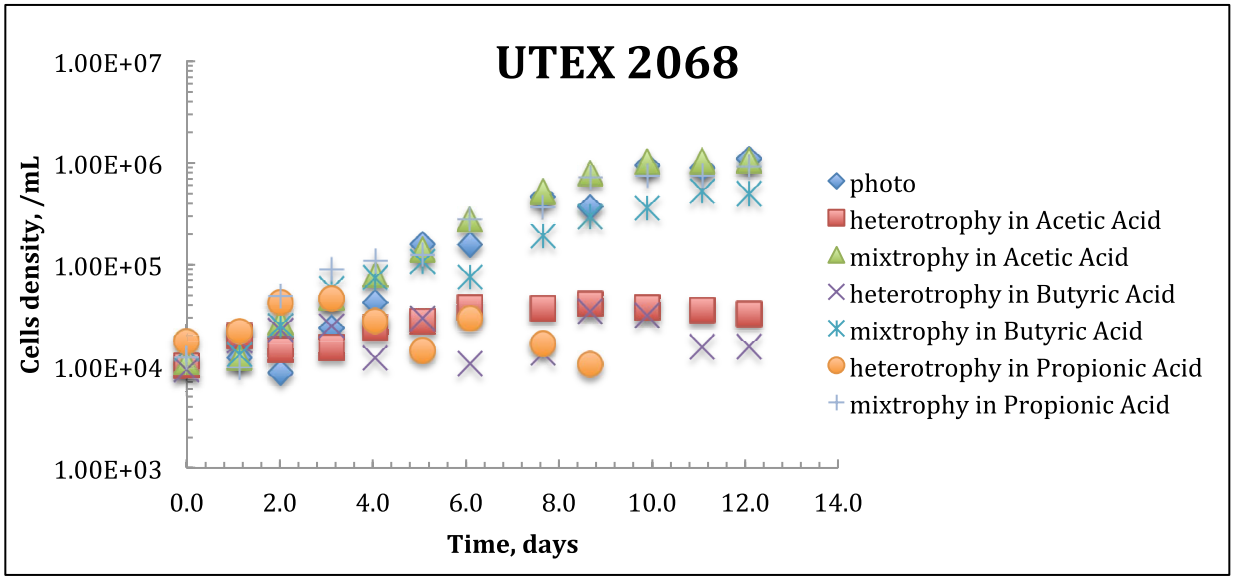

Figure 22. Growth curves for UTEX 2068

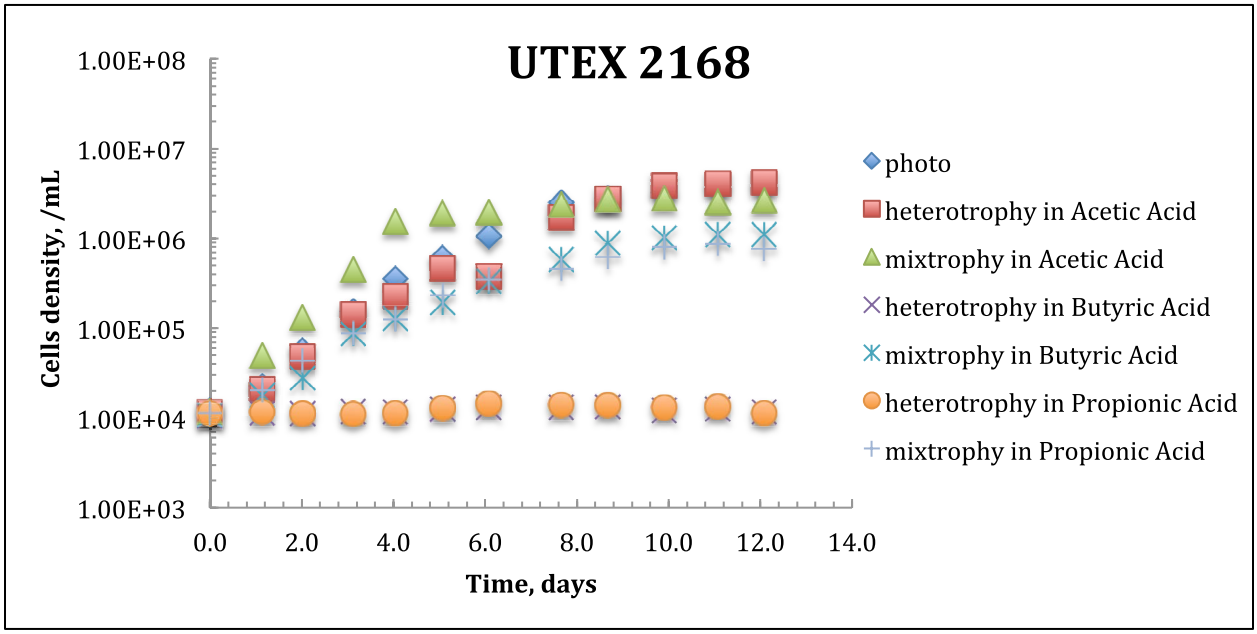

Figure 23. Growth curves for UTEX 2168

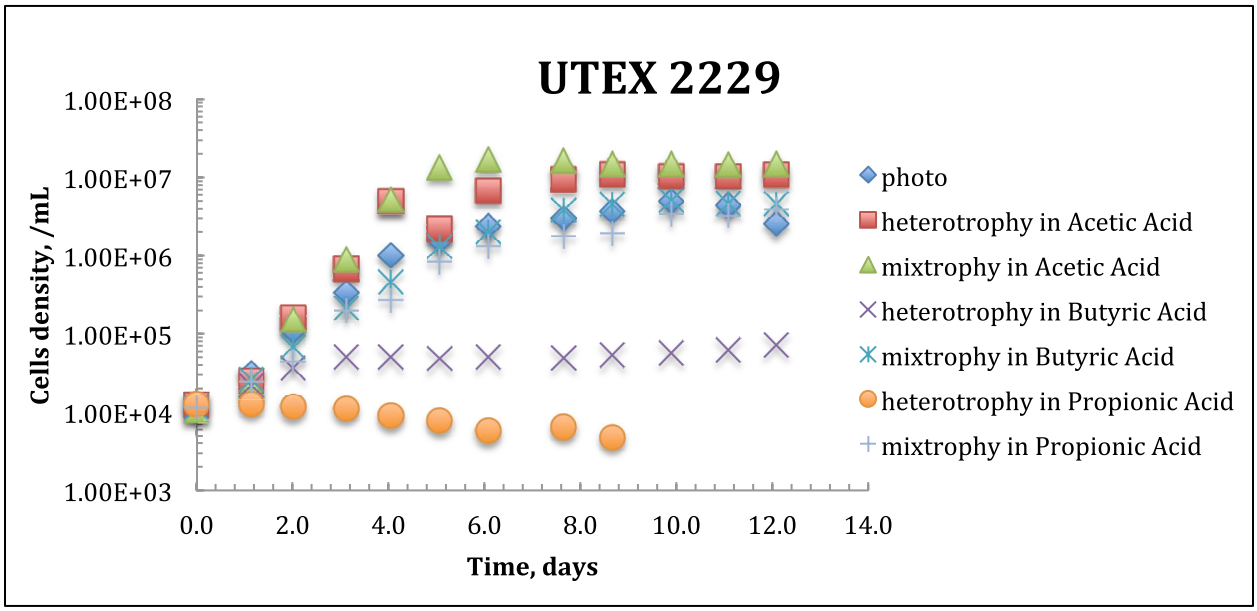

Figure 24. Growth curves for UTEX 2229

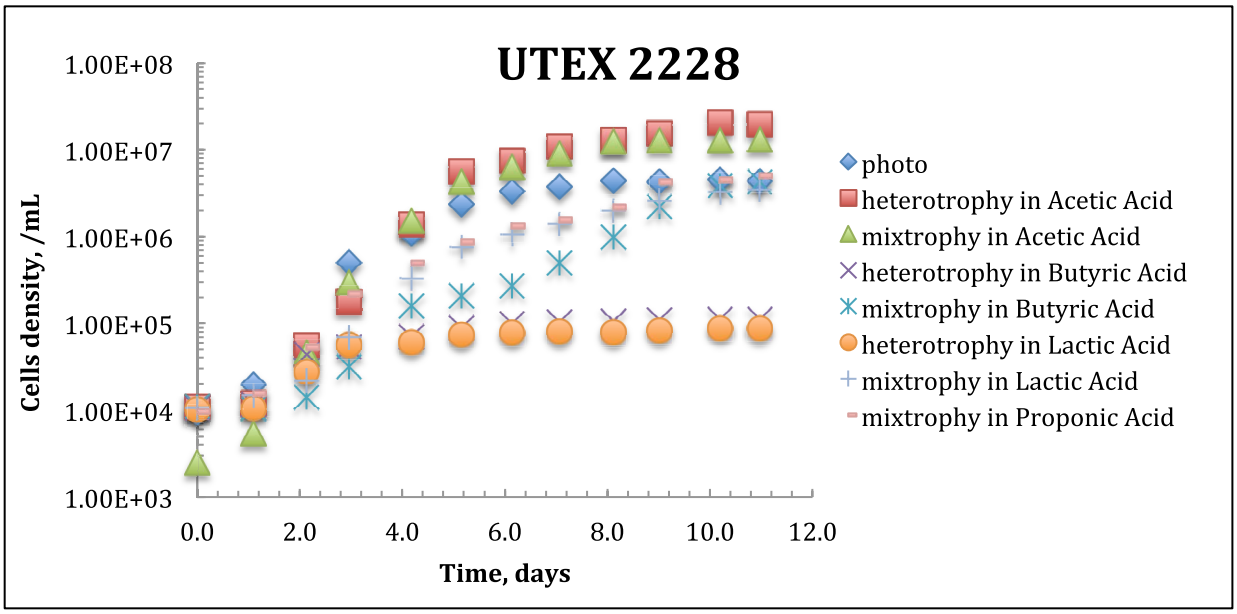

Figure 25. Growth curves for UTEX 2228

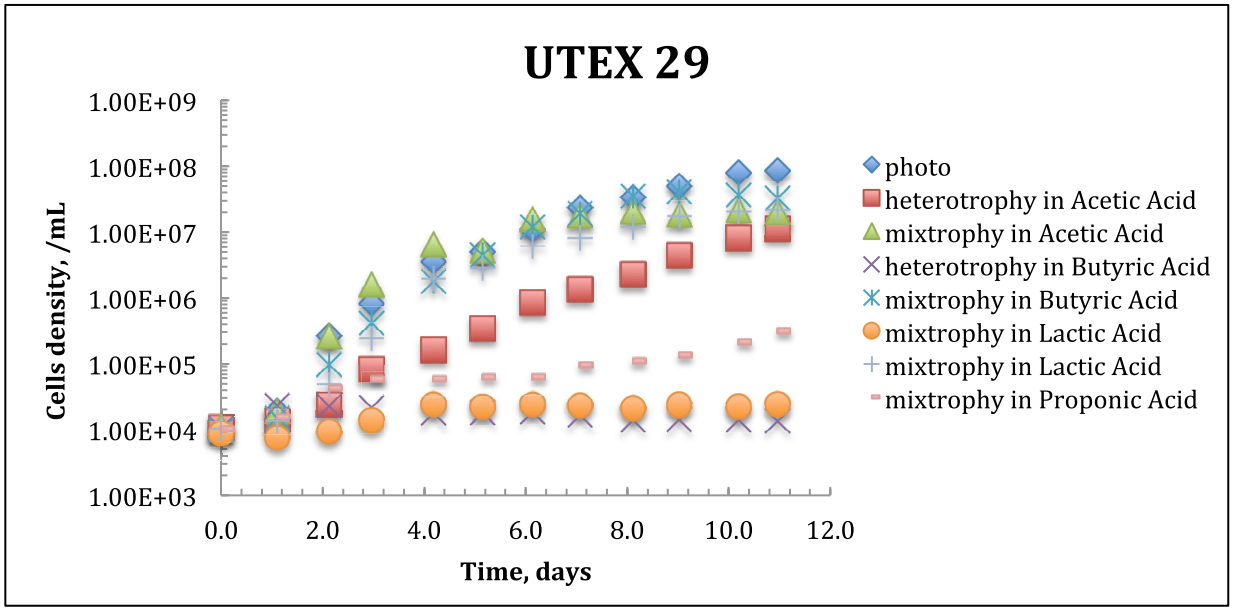

Figure 26. Growth curves for UTEX 29

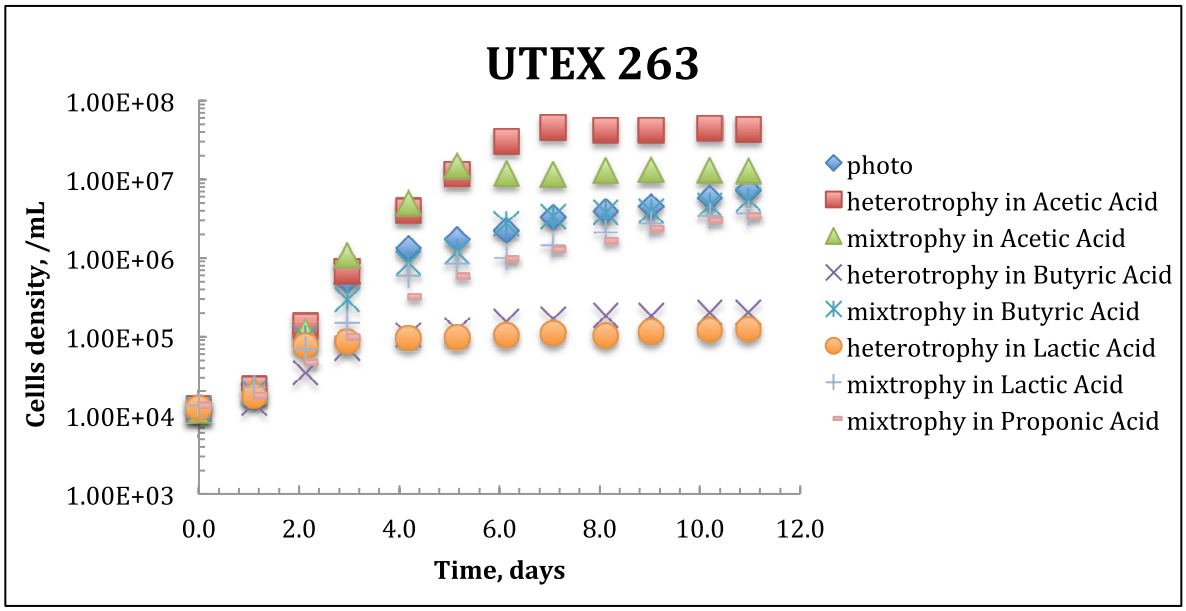

Figure 27. Growth curves for UTEX 263

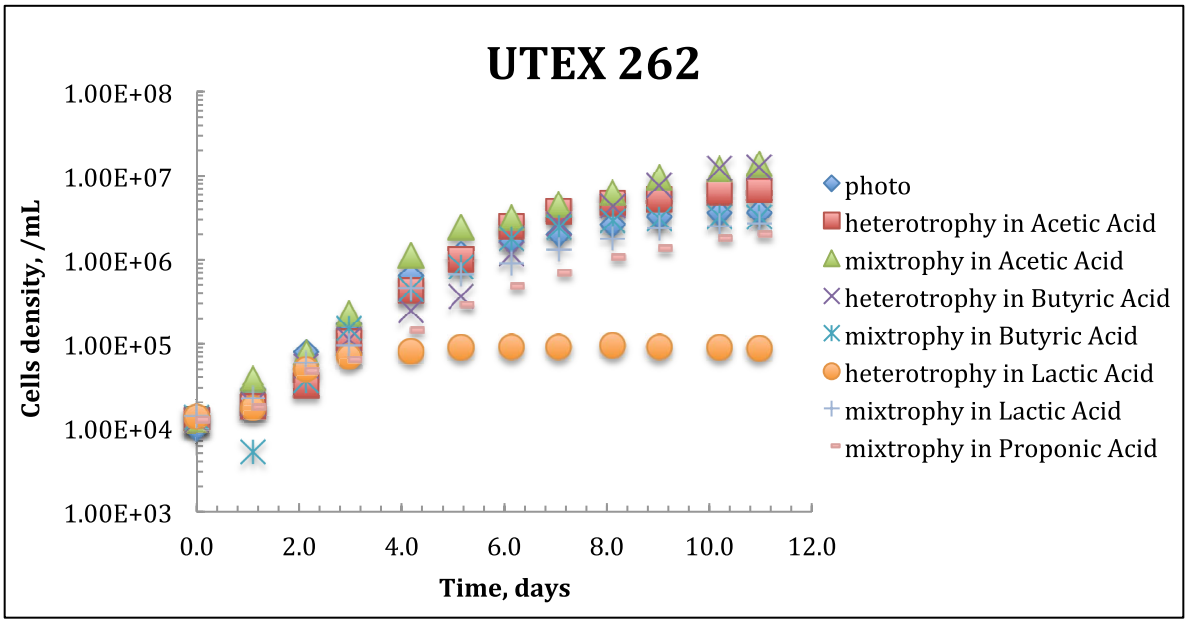

Figure 28. Growth curves for UTEX 262

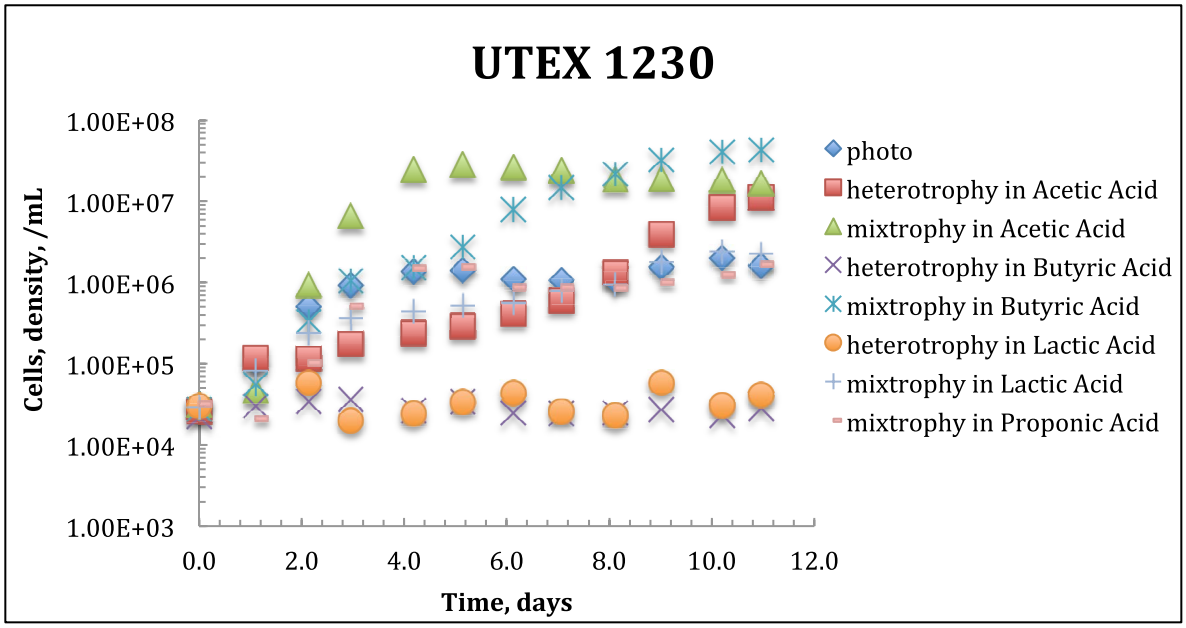

Figure 29. Growth curves for UTEX 1230

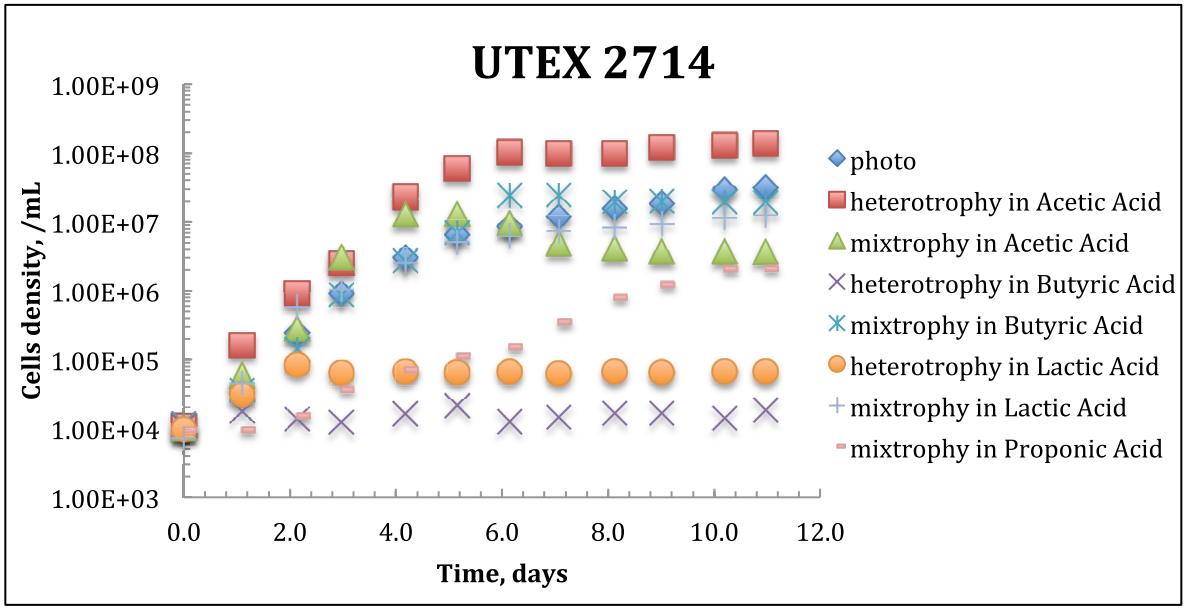

Figure 30. Growth curves for UTEX 2714

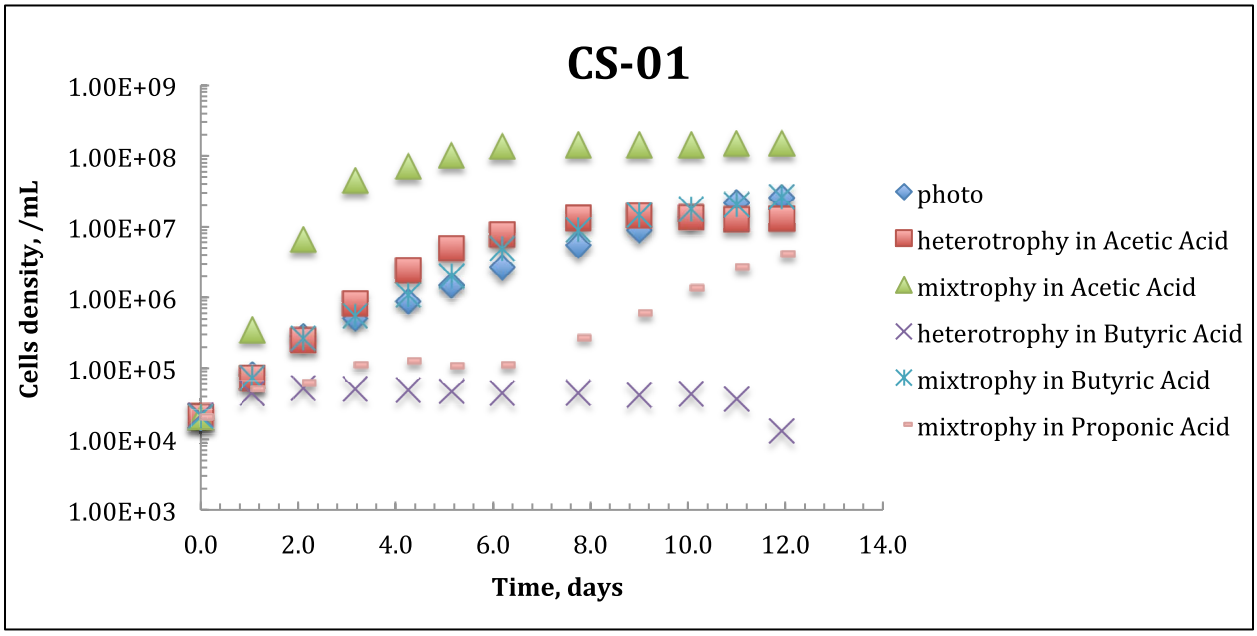

Figure 31. Growth curves for CS-01

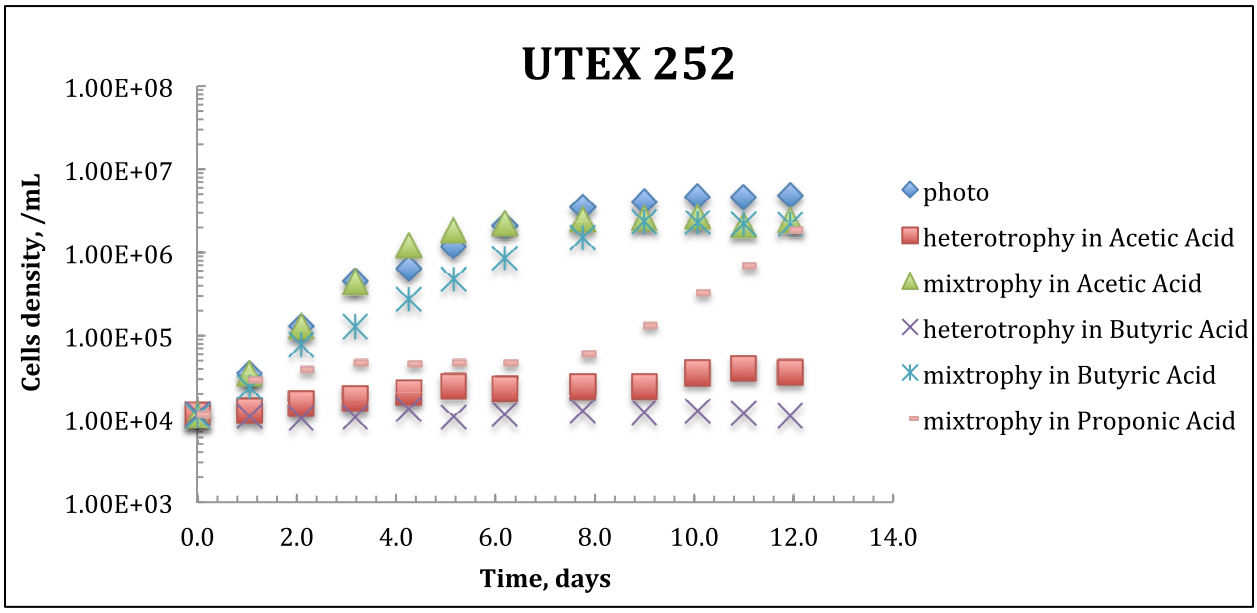

Figure 32. Growth curves for UTEX 252

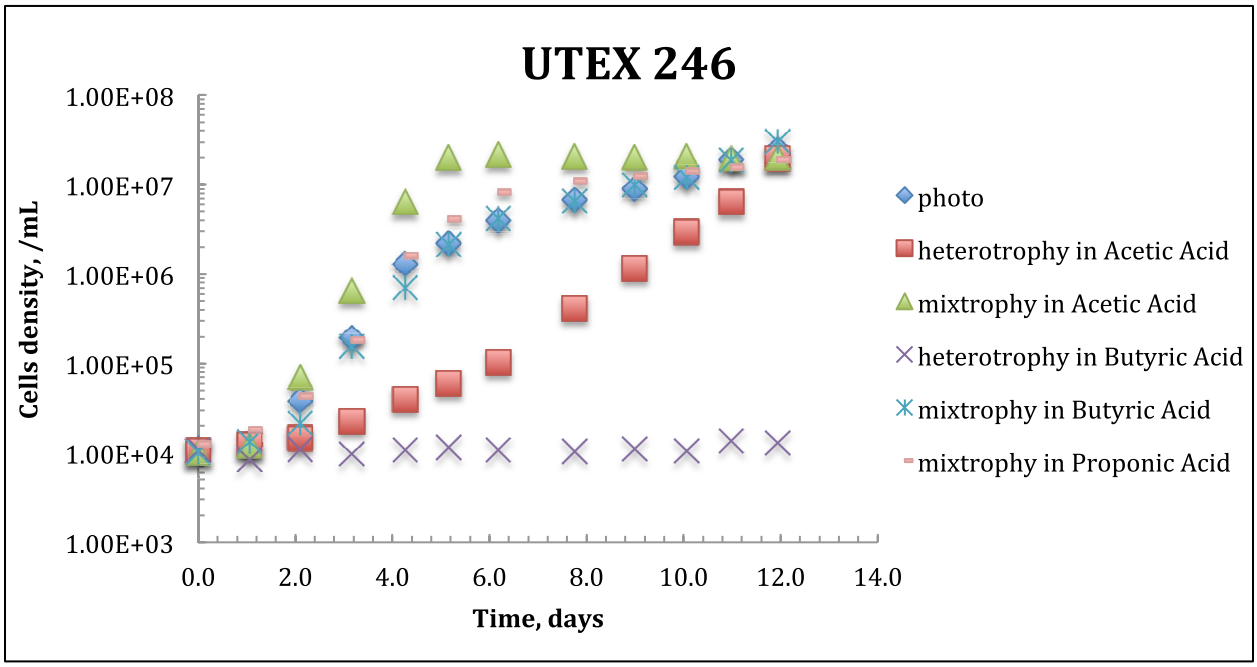

Figure 33. Growth curves for UTEX 246

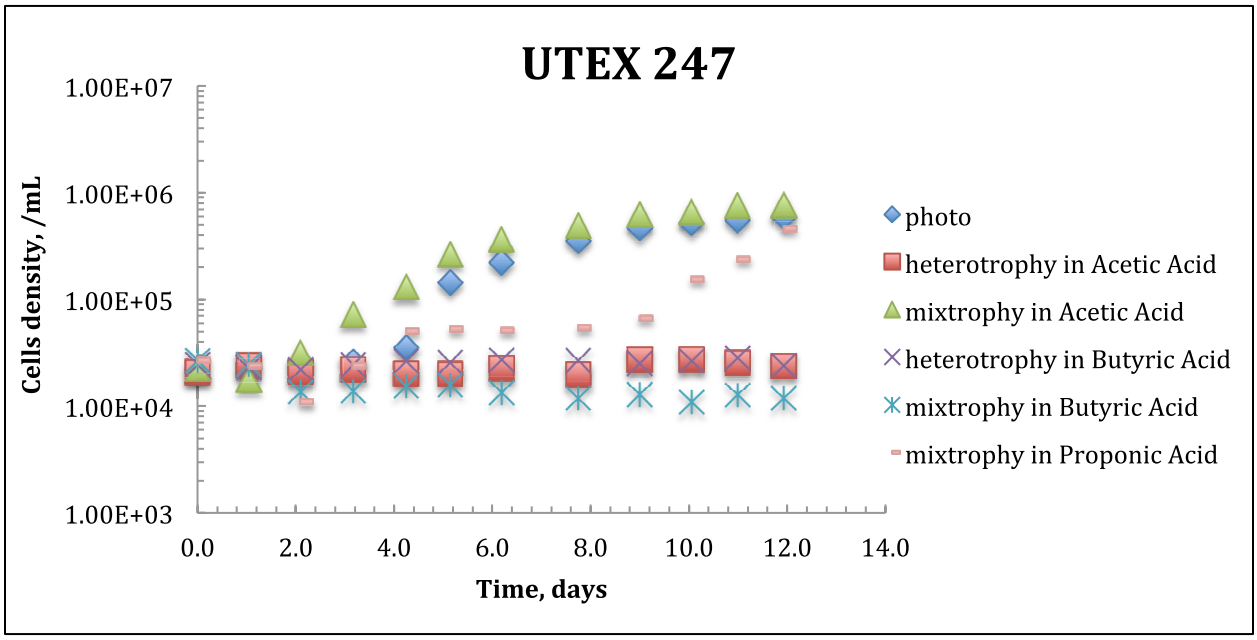

Figure 34. Growth curves for UTEX 247

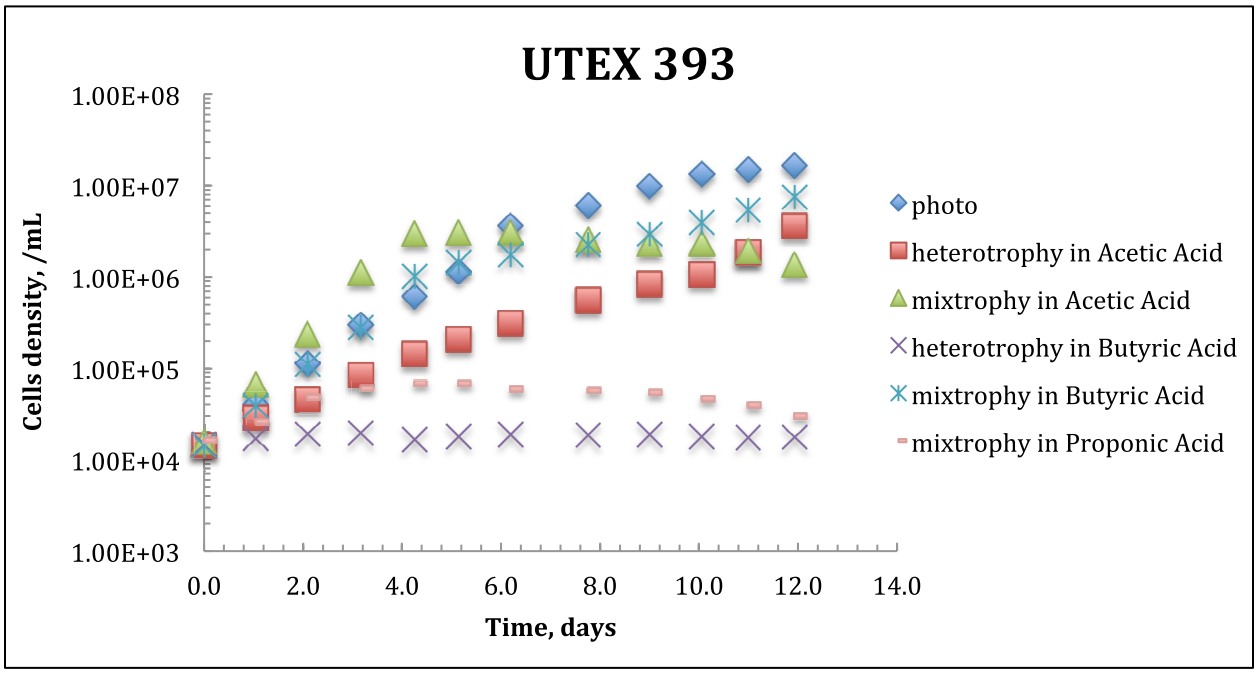

Figure 35. Growth curves for UTEX 393

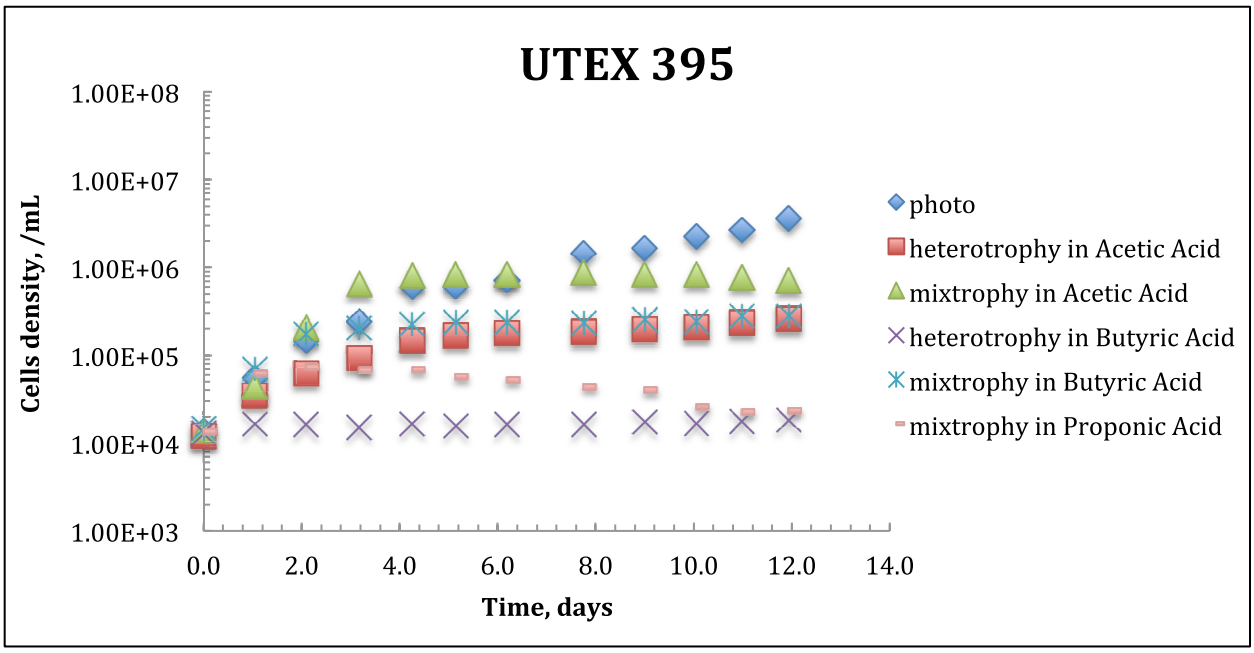

Figure 36. Growth curves for UTEX 395

CHUNYANG SU

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EDUCATION

Johns Hopkins University **Baltimore, MD**
Master of Science in Environmental Engineering Expected May 2014
Cumulative GPA: 3.6/4.0

Donghua University **Shanghai, China**
Bachelor of Science in Environmental Engineering June 2012
Cumulative GPA: 90/100
Awards: National Scholarship from Ministry of Education (Top 1/70); Excellent Student (all years)

RESEARCH EXPERIENCE

Student Researcher, Johns Hopkins University, **Baltimore, MD** July 2013 - Present

- Independently conducted experiments on algae cultivation and screening work and analyzed data collected from previous experiments to optimize bioreactor performance.
- Tested availability of different low-cost biomass after pretreatment as feedstock to cultivate algae and analyzed nutrient content including nitrogen, phosphorus and sugars.
- Implemented the use of pretreated low-priced biomass to cultivate algae, which increased productivity in algae cells density.
- Provided key information on and became the co-author of an abstract entitled "Sourcing organic carbon and non-carbon nutrients for enhanced algal cultivation from low-cost biomass" which has been accepted for poster presentation at the 4th International Conference on Algal Biomass, Biofuels & Bioproducts.

Project Leader, Donghua University, **Shanghai, China** November 2010 - May 2011

- Conducted experiments of textile dyes adsorption onto modified graphite from aqueous media.
- Key player in the integration of team members' research through statistical analysis, contributing significantly to the scientific knowledge of the team.
- Completed a project report in efforts of all team members.
- Published a paper entitled "Kinetics and Thermodynamics Studies of Methyl Orange Adsorption onto PEG-Grafted Expanded Graphite from Aqueous Media" accepted by EPPH - iCBBE 2012.

PROFESSIONAL EXPERIENCE

Shanghai World Exposition **Shanghai, China**
On-site Volunteer Leader May - October 2010

- Positively responded to the main idea "Better City, Better Life" of Shanghai Expo by organizing a series of activities, including collection of recycled materials and guiding passengers to get on and off metro lines.
- Developed and implemented a 25-point survey to ensure readiness for working at service sites.
- Played a key role in the optimization of teamwork, helping them to provide better service as interpreters, receptionists, guides, and media coordinators during the Expo.
- Assigned and evaluated group members performance. Daily report of volunteering was required to help group members to evaluate themselves better, thus to maximize their strengths and avoid weakness.

China Daily **Shanghai, China**
Campus Sales leader June - September 2009

- Received training throughout the year before summer jobs started and selected from multiple candidates, successfully securing the single position on campus for this newspaper company and responsible for the whole college town area.

- Lead a crew up to 15 members to provide door to door newspaper sales promotion and conducted regular sales training sessions to ensure thorough understanding of products, resulting in assisting with communication with student clients.
- Recognized for excellent interpersonal skills. Dealt with student clients in a positive, patient and encouraging manner, increasing sales by 10% compared with prior year.

SKILLS

- **Technical Skills:** Excel; Powerpoint; AutoCAD; C Language Programming (entry-level)
- **Language Skills:** Native Speaker and Writer of Mandarin Chinese; Fluent Speaker and Writer of English; Beginner of Japanese